Cellular and Molecular Characterisation of a Novel Murine Erythroleukaemia System

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This thesis is submitted in part fulfilment of the degree of Doctor of Philosophy in the University of Glasgow

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July 1997

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To Granny, Mum and Dad, Fan, Huahua, and everyone else who loves and cares for me

Acknowledgements

First of all, I would like to thank my supervisor, Dr. Paul Harrison, for his assistance in completing this thesis and constant support during those difficult moments, and also my advisor, Dr. John Pitts, for the fruitful disscusion over the past three years. Dr. Dave Gillespie and Dr. John Frampton have also contributed enormously in data analysis and discussion. Thanks must go to everyone in R4, whose kindness and humour make it such a friendly and enjoyable place to work in, and worth-mentioning is Jim O'Prey for his technical assistance and useful discussion, as well as his endless "wind-ups", throughout the years. I would also like to thank Keith Vass, Peter McHardy and Jian-hua Mao for their patient computing assistance, and Jing-de Zhu for his help and support. A big thank-you also goes to all my great friends, Pierre, Nathalie, Marie, Angeliki, Adam, Len, Kevin, Julie, Eric, Mao-mao, Bao-mei, for all the moral support, "cheer-ups" and great times, and their willingness of being objects of teasing which certainly brightens up those struggling days of my Ph.D.

This thesis would not have existed without my family, in particular, granny, mum and dad, who provide me endless love and support, and a not-too-bad complement of genes. These, and their own hardwork and the will to succeed, are my inspirations now, and for ever and ever. Finally, but not the least, I would like to thank Graeme for his support, understanding and tolerance in the past 8 months, who also provides me a shelter, brilliantly-cooked roast ducks, among other numerous wonderful things.

The work presented in this thesis is sponsored by the Cancer Research Campaign and the Beatson Institute.

Abstract

The ELM erythroleukaemia system is unusual in that the erythroleukaemia cells growing *in vivo* in the spleen cannot be maintained *in vitro* except in contact with bone marrow-derived stromal cells. They are also unusual in that they retain the ability to undergo erythroid differentiation in response to physiological growth factors, such as Epo and IL-3. Clonal stroma-dependent ELM cells (ELM-D) have been isolated in the presence of stroma. By selecting for growth of ELM-D cells without stroma, a series of stroma-independent clones (ELM-Is) have also been obtained. They are more tumorigenic when injected into mice compared with their stroma-dependent parent. All of the stroma-independent clones, apart from one (i.e. ELM-I/1), are still able to differentiate into mature erythroid cells in response to Epo or IL-3. This particular cell line, that is blocked in differentiation, also displays the highest tumorigenicity *in vivo*. The ELM system therefore provides a unique opportunity to analyse the molecular nature of stromal control of erythropoiesis, and identify genetic events involved in confering stroma-independent growth, differentiation arrest and increased tumorigenicity.

The work presented in this thesis has demonstrated that, upon stroma-withdrawal, the majority of the ELM-D population dies out with characteristics of apoptosis. However, the fact, that α-*globin* expression in ELM-D cells is largely increased when the stroma is removed, indicates that ELM-D cells undergo erythroid differentiation in the absence of the stroma. Therefore, at least one mechanism whereby the stromal cells maintain the long-term growth of ELM-D cells is to prevent them from further maturation.

Gene expression and functional studies of the ELM system have revealed the following:

(1) The stroma-independent cells display an elevated expression of an *ets* oncogene, *erg*, at the mRNA level in comparison to ELM-D cells. However, only ELM-I/1 cells seem to express detectable level of the *Erg* protein.

(2) The expression of another *ets* oncogene, *fli-1*, in the ELM-I/1 cells is significantly activated at both mRNA and protein levels, but is absent in all the other ELM cells;

(3) The *bcl-2* expression at the mRNA level in the stroma-independent cells is higher than that in ELM-D cells, especially in ELM-I/1 which is the only cell line wherein the *Bcl-2* protein is expressed at a detectable level. Functional analysis indicate that over-expression of *bcl-2* is not sufficient to induce differentiation arrest or increased tumorigenicity in ELM-I cells.

Finally, transient transfection assays have demonstrated that the *Fli-1* protein can transactivate a partial *bcl-2* promoter in a quail fibroblast cell line. The *bcl-2* promoter activity is higher in ELM-I/1 cells, which express high levels of *Fli-1* protein, than that in ELM-I/2 cells, which do not express *fli-1* at all. This implies that *Fli-1* may be responsible for the up-regulation of *bcl-2* expression in ELM-I/1 cells. DNase I footprinting analysis has identified six binding sites for the ELM nuclear proteins within the minimum *bcl-2* promoter region. However, gel retardation assays and "supershift" analysis using a *Fli-1*-specific antibody do not reveal the binding of *Fli-1* to any of the six nuclear protein binding sites. The tentative conclusion is, therefore, that *Fli-1* may transactivate the *bcl-2* promoter by an indirect mechanism.

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Abbreviations

A	Adenosine
ALL	Acute lymphoblastic leukaemia
ATD	Amino-terminal transactivation domain
α-MEM	Alpha minimal essential medium
APS	Ammonium persulphate
BFU-E	Burst-forming unit-erythroid
β-gal	β-galactosidase
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
°C	Degree centigrade
c-	Cellular
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
C.E.	Cloning efficiency
c/EBP	CCAAT/enhancer-binding protein
CFU-E	Colony-forming unit-erythroid
$CHCl_3$	Chloroform
Ci	Curie
CML	Chronic myelomonocytic leukaemia
CSF-1	Colony-stimulation factor-1
CTD	Carboxy-terminal transactivation domain
DEPC	Diethylpyrocarbonate
dH2O	Distilled water
ddH2O	Double distilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	3' deoxyribonucleoside 5' triphosphate
DTT	Dithiothreitol
EBD	*Ets*-binding domain
EBS	Ets-binding sites
ECM	Extracellular matrix
EDTA	Ethylenediaminetetra-acetic acid, disodium salt

EGF	Epidermal growth factor
ELM-D	Stroma-dependent ELM erythroleukaemia cell
ELM-I	Stroma-independent ELM erythroleukaemia cell
env	Envelope
Epo	Erythropoietin
Epo-R	Erythropoietin receptor
ERSR	*Erg*-specific region
FLSR	*Fli-1*-specific region
FN	Fibronectin
FP	Footprint
F-MuLV	Friend murine leukaemia virus
ΦOH	Phenol
FGRB	Formaldehyde gel running buffer
FV	Friend virus
FV-A	Friend virus (anaemia-inducing strain)
FV-P	Friend virus (polycythaemia-inducing strain)
g	Gram
G	Guanine
gag	Group antigen
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
h	hour
HBS	Hepes-buffered saline
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid)
HGF	Haemopoietic growth factor
HLH	Helix-loop-helix
HM	Haemopoietic microenvironment
ICE	Interleukin-1β converting enzyme
IFN	Interferon
IGF-1	Insulin growth factor-1
IPTG	Isopropylthiogalactoside
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-3	Interleukin-3
IL-11	Interleukin-11
KAc	Potassium acetate
k	Kilo
kD	Kilo dalton
l	Litre
LB	L-broth

LTBMC	Long-term bone marrow culture
LTR	Long terminal repeat
μ	Micro
m	Milli
M	Molar
mA	Milliamps
MAP kinase	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MEL	Murine erythroleukaemia cells
min	Minute
MIP-1α	Macrophage inhibitory protein-1α
MOPS	3-(N-morpholino) propanesulphonic acid
mRNA	Messenger ribonucleic acid
mSCF	Transmembrane form of stem cell factor
n	Nano
NaAc	Sodium acetate
NGF	Nerve growth factor
NRE	Negative regulatory region
ONPG	o-Nitrophenyl β-D-galactopyranoside
p	Pico
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered-saline
PCI	Phenol:chloroform:isoamylalcohol
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PGTB	Protein-gel tank buffer
PKC	Protein kinase C
pmol	Picomole
PMSF	Phenylmethylsulfonyl fluoride
poly(dI-dC)	Polydeoxyinosinic deoxycytidylic acid
Rb	Retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
sec	Seconds
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate

SFFV	Spleen focus-forming virus
Sl	*Steel* locus
Sl^d	*Steel-Dicke* locus
Sp1	Promoter-specific transcription factor-1
SRE	Serum-response element
SRF	Serum-response factor
T	Thymine
TB	"Terrific" broth
TBS	Tris-buffered saline
TEMED	Tetramethylenediamine
TGF-β	Transforming growth factor-β
tk	Thymidine kinase
TNF-α	Tumour necrosis factor-α
TNT	Coupled *in vitro* transcription and translation reactions
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol
tRNA	Transfer ribonucleic acid
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Units
UTR	Un-translated region
UV	Ultra violet
V	Volts
v-	Viral
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
v/v	Volume for volume
W	Watts
W	*White-spotting* locus
w/v	Weight for volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactpside

Throughout this thesis, genes and alleles are indicated by italics with the first letter in a small case, e.g. *bcl-2*, while proteins are indicated by italics with the first letter in a capital case, e.g. *Bcl-2*.

PART I: INTRODUCTION

CHAPTER 1: Haemopoiesis

Human peripheral blood constitutes 7% of body weight and about one-half blood volume is occupied by erythrocytes. Since erythrocytes and other blood cells have an average life span of 4 months or less, it has been estimated that, in human, approximately 3.7 x 10^{11} blood cells need to be produced per day to replace those lost by natural wastage. Injury, infection and haemotological stress increase this demand for cell production further. This generation of blood cells - haemopoeisis - is a multi-step process involving extraordinary self-renewal of rare haemopoietic stem cells, proliferative expansion of multi-lineage progenitor cells, progressive commitment of progenitors to single lineage differentiation, and consequent positioning of the cells within the tissue.

Regulation of haemopoiesis is mediated, at least in part, by a range of stimulatory and inhibitory growth factors that influence the haemopoietic stem cells, their more developmentally restricted progeny, or both. These include interleukins-1 to -11 (IL-1 to IL-11), haemopoietic cell colony-stimulating factors (CSFs), erythropoietin (Epo), thrombopoietin, stem cell factor (SCF, also known as c-kit ligand or mast cell growth factor), transforming growth factor β (TGF-β), macrophage inflammatory protein 1α (MIP1-α), and various other growth factors such as the interferons (IFNs) and insulin-like growth factors (IGFs) (Whetton and Dexter, 1993; D'Andrea, 1994). Many of these growth factors have overlapping biological activities and target cells, and different combinations of growth factors seem to have different biological effects.

In addition, the haemopoietic microenvironment (HM), especially stromal cells, also plays a pivotal role (Zipori and Tamir, 1989; Zipori, 1992). At the sites of haemopoiesis, that is, bone marrow, spleen and foetal liver, proliferation and differentiation of haemopoietic stem and progenitor cells occur in intimate contact with the stromal cells and the associated extracellular matrix (ECM). Electron microscopy revealed intimate interactions between haemopoietic cells and the stromal cells, which form secluded niches within HM. Although the exact role of the HM in determining haemopoietic stem and progenitor cell behaviour is not fully known, multiple possible functions have been theorised. As currently envisioned, the HM provides direct cell-cell contact between supporting and haemopoietic cells, provides anchorage for both growth factors and haemopoietic cells, supplies specific positive and negative growth regulatory factors, and probably provides for multiple cellular communications within what we have termed a "local area network". Within this network, stromal cells probably play a key role by both producing growth regulatory proteins and secreting complex ECM proteins for stabilisation of growth factors in high local concentrations within the adherent layer

of haemopoietic stem and progenitor cells. In particular, sequestration of growth factors on ECM components of stromal tissues and expression of membrane forms of growth factors may provide the basis for the formation of microenvironments (Zipori, 1992). The locally concentrated presentation of growth factors at the adherent stromal cell surface is thought to prime the stem and progenitors cells to respond to combinations of other local or circulating factors at physiologically relevant concentrations.

Relatively little is known about the differentiation-inducing activity of haemopoietic growth factors (HGFs) or microenvironment but two plausible models have been proposed, the instructive and stochastic models (D'Andrea, 1994), which might not necessarily be mutually exclusive, as agreed by the hybrid model, and exactly which model dominates may depend on cellular context, differentiation stage of the cells, the presence of other factors. The instructive model argues that binding of the HGFs to their receptors in combination with other components of the cellular microenvironment, such as cell adhesion molecules and (ECM), acts as an inducer of differentiation and determines lineage choice from multi-potent cells, presumably by influencing gene expression. In contrast, the stochastic model are based on the premise that multi-potent cells do not require exposure to external inducing stimuli to express the genetic changes that are presumably required for lineage restriction and argues that HGFs and HM simply permit the proliferation and development of these intrinsically committed cells and the subsequent expression of the mature cell phenotype.

In either model, HGFs and HM are proposed to act through a series of secondary messengers which transduce signals into the nucleus that lead to initiation of differentiation programs and co-ordinated, lineage-specific gene expression. Therefore, key transcription factors, including *c-Myb*, *GATA-1*, *GATA-2*, *SCL/Tal-1*, *Rbtn2/LMO2*, *Ikaros*, *Pax-5*, *PU.1*, *NF-E2* and *EKLF*, are essential for multiple aspects of haemopoietic development - from survival, proliferation, lineage commitment through terminal maturation (Orkin, 1995; Shivdasani *et al.*, 1996; also see Section 2.2).

CHAPTER 2: Erythropoiesis and "Erythroid-specific" Transcriptional Regulators

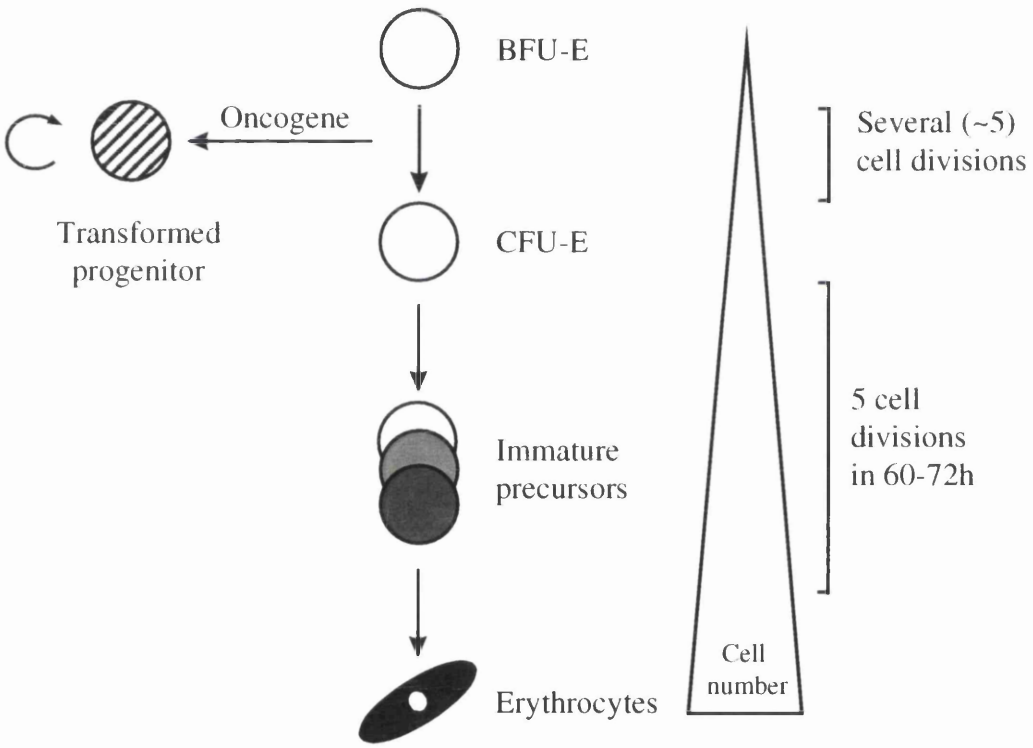
2.1. Erythropoiesis.

The earliest committed erythroid cell is a lineage-restricted product of a myeloid precursor, a cell derived from a pluripotent stem cell that has the potential to generate all other myeloid cells. There are two distinct types of committed erythroid progenitors, initially defined by the type of colony derived from such immature cells in semisolid methocel medium or fibrin clots. They are termed colony forming unit-erythroid (CFU-E), the mature progenitor, and burst forming unit-erythroid (BFU-E), the more primitive one (Figure 2.1). However, these two groups of cells probably represent two extremes of a continuum of development from the earliest unipotent progenitor cell to the mature erythrocyte. The CFU-E gives rise to a small colony of 10-50 mature, haemoglobinised cells within 2-3 days. On the other hand, the BFU-E develops into diffuse colony of tens to hundreds of CFU-E, each of which then forms a compact colony, resulting in "burst-like" colonies containing thousands to tens of thousands of mature erythrocytes after 6-10 days. Under defined conditions, these two types of colonies appear with highly reproducible kinetics. This suggests that erythroid differentiation is a rigid program that involves a fixed number of cell divisions and, at the CFU-E stage, leads to dramatic, complex changes in gene expression as well as in cell shape and function (reviewed by Beug *et al.*, 1994).

Numerous studies over the years have yielded substantial insights into the molecular mechanisms whereby haemopoietic growth factors (HGFs) regulate erythroid differentiation. Using highly purified human CFU-E and BFU-E, it was shown that CFU-E require erythropoietin (Epo) and insulin-like growth factor 1 (IGF-1) and/or insulin for colony formation in fibrin clots (Sawada *et al.*, 1989); whereas BFU-E require human stem cell factor (SCF), the ligand for the receptor tyrosine kinase *c-Kit*, for growth and differentiation in culture (Dai *et al.*, 1991). Studies employing purified BFU-E indicated that these cells are able to "count" generations (Sawada *et al.*, 1991; also see Figure 2.1): when the progenitors were first cultivated in liquid medium for increasing time periods and then assayed in fibrin clots for their remaining cell division capacity, the size of erythroid colonies obtained was inversely proportional to the number of cell doublings in suspension culture prior to colony formation in the fibrin clots. This, and work by others (Beug *et al.*, 1994), indicates that erythroid differentiation involves co-ordinated re-programming of gene transcription and specific alterations in cell cycle control. Upon differentiation induction, the CFU-E cells undergo five cell divisions during the first three days of differentiation and then arrest in G1

Figure 2.1. Current view of normal erythroid differentiation as revealed by bone marrow colony assays and work on purified progenitors.

Two types of morphologically un-differentiated progenitors, the early BFU-E and its descendant, the more mature CFU-E, differentiate into post-mitotic erythrocytes via partially differentiated intermediates. The continuous increase in cell number, and the fact that both differentiation of BFU-E into CFU-E and terminal differentiation of the latter may involve a strict cell division program able to "count" cell generations, are indicated at the right. The ability of retroviral oncogenes to induce continuous self-renewal (circular arrow) is also depicted (adapted from Beug *et al.*, 1994).



(Beug *et al.*, 1994). The final steps in the development of fully mature erythrocytes proceed in the absence of further cell divisions. Two important and active events occur in the first 16-24h after induction of differentiation. Firstly, the gene expression pattern of the cells was extensively re-programmed. The expression of genes characteristic of self-renewing cells (e.g. *c-myb*, *c-kit* and the oestrogen receptor gene) abruptly ceases, whereas expression of erythroid-specific transcription factors, such as *GATA-1*, *GATA-2*, *SCL*, *NF-E2* (disccused in detail in Section 2.2), is strongly up-regulated, followed slightly later by the activation of numerous genes encoding late erythrocyte proteins (Beug *et al.*, 1994). Secondly, the cell proliferation rate increases drastically, as indicated by a reduction of cell cycle length from about 20 to 12h, which is almost entirely due to a shortening of the G1 period from 12 to 5 h, while S and G2 phase lengths are not affected (Beug *et al.*, 1994). Contraction of cell cycle is accompanied by a decrease in cell volume from about 300 to less than 70 femtolitres. Measurements of amino acid incorporation versus cell size showed that the rate of protein synthesis normalised to cell volume remains constant (Beug *et al.*, 1994). Thus cell size reduction seems to be a necessary consequence of G1 shortening in this case, as reduced production of cellular mass due to the shorter G1 phase could not be compensated for by a higher rate of protein synthesis. It is tempting to speculate that the progressive size reduction undergone by the maturing cells during the five "terminal differentiation divisions" is one of the mechanisms by which the differentiating cells "count" cell divisions (perhaps similar to those found in early embryogenesis). All these findings provide the first tentative evidence that erythroid differentiation, like myoblast differentiation, may involve control by a "master switch", which not only completely re-programs the pattern of gene expression, but also profoundly alters cell cycle control. It is logic to consider the possible existence of "master switch" as it is unlikely that HGFs regulate directly every details of commitment and/or differentiation sequence. They may merely initiate a self-sustaining genetic programme which is intrinsically determined by the cells of question.

In addition to HGFs, the microenvironment created by stromal cells is also essential for erythropoiesis (Zipori and Tamir, 1989; Zipori, 1992; also see Chapter 1). A series of studies carried out in Dr. M. Obinata's laboratory demonstrated that stromal endothelial (Yanai *et al.*, 1989; Yanai *et al.*, 1991) and epithelial-like cell lines (Ohneda *et al.*, 1990), derived from mouse spleen and foetal liver, respectively, are able to support large erythroid colony formation and mimic erythropoiesis *in vivo*. The resulting mature erythrocytes and erythroblasts showing cytoplasmic budding were present in the large erythroid colonies generated on the stromal cell lines. This kind of *in vitro* microenvironment seems to be created by direct contact and/or short range communication between the erythroid progenitor cells and the stromal cell layer since large erythroid colonies were not formed if the progenitor cells were separated from the

stromal cells by a diffusion chamber or a nucleopore filter. Supplementing with conditioned medium from the stromal cells did not surpport the colony formation, nor did various combinations of soluble growth factors such as IL-3, GM-CFS and Epo. The study was extended by examining the roles of adhesion molecules in erythropoiesis using blocking antibodies (Yanai *et al.*, 1994). These adhesion molecules include very late antigen-4 and -5 (VLA-4 and VLA-5 integrins) and the ligands for VLA-4, vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (FN). The data demonstrated that, although all of them can mediate adhesion of the erythroid progenitors cells to the stroma, formation of large erythroid colonies is only inhibited by the blocking antibodies against VLA-4 and VCAM-1. This implies that collaborative interaction between VLA-4 and VCAM-1 may be important in providing adequate signalling for the erythroid progenitor cells in the erythropoietic microenvironment. Consistent with this conclusion is the recent finding by Hamamura *et al.* (1996) who performed in utero treatment of mice with an anti-VLA-4 monoclonal antibody. They found that, although all haemopoietic cells in foetal liver expressed VLA-4, the treatment specifically induced anaemia, and it had no effect on the development of non-erythroid lineage cells, including lymphoids and myeloids. In the treated liver, almost no mature erythroblasts were detected, although erythroid progenitors were present as judged by their ability to form erythroid colonies *in vitro*. These results indicate that VLA-4 plays a critical role in erythropoiesis specifically *in vivo*.

2.2. Transcriptional regulation of erythropoiesis by *GATA-1*, *SCL/Tal-1* and related proteins.

As discussed in Chapter 1, haemopoiesis is the process by which blood cells acquire particular phenotypes as a result of co-ordinated, cell-specific gene expression. The pattern of gene expression within a cell is established, to a large extent, by cell-specific transcription factors that mediate the net effect of the variety of proliferation and differentiation signals which impinge on the cell. Hence, understanding the functions of transcription factors is essential to the study of haemopoeisis. In this section, transcriptional regulation of erythropoiesis by *GATA-1*, *SCL/Tal-1* and the related proteins, such as *LIM*, *Krüpple* and *Id* family proteins, will be discussed (reviewed by Bockamp *et al.*, 1994; Orkin 1995; Shivdasani and Orkin, 1996; Green, 1996).

2.2.1. *GATA-1.*

GATA-1 is the first example of a family of zinc finger proteins that bind to the DNA sequence motif GATA. The *GATA* family proteins share a conserved, cysteine-rich, metal-binding motif, which contains two finger domains and is essential for DNA binding. Structural and functional analysis of *GATA-1* has also revealed a C-terminal

activation domain capable of transactivating reporter constructs containing a GATA motif in fibroblasts (Martin *et al.*, 1990). Within haemopoietic cells, expression of *gata-1* is very abundant in erythroid cells, and is also present in megakaryocyte, eosinophil, mast cell lineages and multipotential progenitors. During differentiation, the *gata-1* mRNA level increases in some lineages including erythoid, whereas it decreases in others including granulocyte and monocytes (Bockamp *et al.*, 1994). Thus, *GATA-1* seems to play a role in differentiation of multiple blood lineages. More persuasive evidence in support of *GATA-1* as a lineage-directing transcription factor has recently been provided by experiments in *Myb-Ets-* or *v-Myc*-transformed myelomonocytic cell lines (Kulessa *et al.*, 1995). Over-expression of *gata-1* in these cell lines reprograms these cells into eosinophils, thromboblasts and erythroblats. *Gata-1*-null embryonic stem (ES) cells do not produce mature erythroid cells when injected into mouse blastocysts, although *gata-1*$^{-/-}$ ES cells in those chimeric mice are fully able to develop into other haemopoietic lineages as well as other tissues (Pevny *et al.*, 1991; Simon *et al.*, 1992). The consequences of absence of *gata-1* have been further defined by *in vitro* differentiation assays, which show a maturation arrest of erythroid precursors at the pro-erythrblast stage followed by apoptosis of these cells (Weiss *et al.*, 1994; Pevny *et al.*, 1995; Weiss and Orkin, 1995). Thus, although a role for GATA-1 in lineage selection is possible, it is the survival and terminal maturation of erythroid precursors that appear to be critically dependent on *GATA-1*. Target genes for *GATA-1* include the *gata-1* gene itself, globin genes, and numerous other genes expressed specifically in erythroid and megakaryocyte lineages. Overall, an emerging theme for *GATA-1* action appears to be that it promotes differentiation both by inducing differentiation-specific genes and by simultaneously suppressing genes involved in cell proliferation.

Modulation of *GATA-1* activity has been shown to be the consequence of exposure to growth factors or differentiation inducers, and this occurs at various levels including transcriptional regulation, re-localisation, phosphorylation, and direct physical interactions. Baron and Farrington (1994) showed that pro-erythroblasts and mature erythroid cells contain a diffusible activity (TAG) capable of transcriptional activation of the *gata-1* gene, and that this activity decreases during terminal differentiation of these erythroid cells. Briegel *et al.* (1996) demonstrated that, in primary erythroid progenitor cells, the *GATA-1* protein is predominantly located in the cytoplasm; while induction of differentiation causes its rapid re-localisation to the nucleus, suggesting that nuclear translocation may constitute an important regulatory step in *GATA-1* activation. Crossley and Orkin (1994) reported that *GATA-1* is phosphorylated on 6 serines within its amino terminus in un-induced murine erythroleukaemia cells (MEL), and a 7th site becomes phosphorylated after the MEL cells are induced to differentiate by DMSO. The significance of the induced phosphorylation is still elusive as it does not influence

DNA-binding affinity or sequence specificity, DNA bending, or transcriptional transactivation by *GATA-1*. Several proteins can modulate *GATA-1* activity by direct physical contact and these include oestrogen receptor (ER) and the *Krüpple* family proteins. Oestrogen is known to suppress erythropoiesis and induce apoptosis in erythroid cell lines *in vitro*. Recent evidence provided by Blobel *et al.* (1995) and Blobel and Orkin (1996) has shed light on the underlying molecular mechanisms. They demonstrated that oestrogen elicits its effects by inhibition of the transcriptional activity of *GATA-1*. Moreover, co-immunoprecipitation experiments demonstrated an association between *GATA-1* and ER in a ligand-dependent manner. Merika and Orkin (1995) reported that *GATA-1* activates transcription in a synergistic fashion with two *Krüpple* family factors, *Sp1* and *EKLF*, by direct protein-protein interaction. Finally, nuclear localisation studies carried out by Elefanty *et al.* (1996) revealed specific bright foci of *GATA-1* within the nucleus in erythroleukaemia cells, primary murine erythroblasts and megakaryocytes, in addition to diffused nucleoplasmic localisation. These foci, which were preferentially found adjacent to nucleoli or at the nuclear periphery, did not represent sites of active transcription or binding of *GATA-1* to consensus sites in the β-globin loci. Interestingly, *GATA-2* and *GATA-3* proteins also localised to the same nuclear bodies in cell lines co-expressing *gata-1*, *gata-2* or *gata-3*. This pattern of distribution is, thus far, unique to the *GATA* transcription factors and suggests a protein-protein interaction with components of the nuclear bodies via the *GATA* zinc finger domain.

2.2.2. *SCL/Tal-1*.

The *scl/tal-1* (hereafter called *scl*) gene was originally identified at the breakpoint of a chromosomal translocation in T-cell acute lymphoblastic leukaemia (Chen *et al.*, 1990). Since then, three other related genes have been identified, *LYL-1*, *NSCL* and *tal-2*, all of which encode transcription factors that share a conserved basic helix-loop-helix (bHLH) motif. The *scl* expression is largely restricted to haemopoietic tissues, although some expression is seen in adult and developing brain and in endothelial cells. Within the haemopoietic system, *scl* is expressed in primitive multi-potent progenitors as well as in cells committed to the erythroid, mast, megakaryocytic lineages, but it is not found in several other cell types including T-cells, mature B-cells, or in many cell lines with granulocyte or monocyte characteristics.

Many lines of early evidence have implicated *SCL* as a positive regulator of erythroid differentiation and demonstrated that quantitative changes in the *scl* expression occur during normal erythroid and granulocyte/monocyte differentiation. For example, HMBA-induced erythroid differentiation of MEL cells results in biphasic modulation of the *scl* expression, with an early transient fall and a late rise in the *scl* mRNA (Green *et*

al., 1992). In contrast, HMBA-induced myeloid differentiation of K562 cells is accompanied by an early transient fall in the *scl* mRNA, which was initially recovered but subsequently followed by a late persistent down-regulation (Green *et al.*, 1993). Similarly, Epo-induced erythroid differentiation of FDCPmix A4 cells results in up-regulation of the *scl* mRNA, whereas growth factor-induced granulocyte/monocyte differentiation is accompanied by a marked decrease in the *scl* mRNA (Cross *et al.*, 1994). Studies of the *scl* expression at the protein level in MEL cells during differentiation indicate that an early transient fall in the *SCL* protein is not required for erythroid differentiation (Murell *et al.*, 1995). Support for this concept has come from experiments involving over-expression of sense and antisense *scl* constructs. Exogenous expression of *scl* inhibits myeloid differentiation of M1 cells (Tanigawa *et al.*, 1993) but enhances erythroid differentiation of MEL cells (Aplan *et al.*, 1992). Moreover, stable expression of antisense *scl* constructs inhibits erythroid differentiation of MEL cells (Aplan *et al.*, 1992).

In addition, more recent data have also suggested a regulatory role of *SCL* in proliferation and anti-apoptosis. Leroy-Viard *et al.* (1995) reported a truncated form of *SCL* protein in a sub-clone of Jurkat T-cell line, which has a dramatically decreased DNA-binding activity to the *SCL* consensus sequence. It was found that the sub-clone exhibits premature apoptosis upon medium depletion or serum reduction when compared with the parental cells. Stable expression of the mutant *SCL* protein in Jurkat cells results in a phenotype that is similar to that of the mutant Jurkat subline, indicating that the truncated *SCL* protein behaves like a dominant negative mutant. Furthermore, Condorelli *et al.* (1997) showed that in late myeloid progenitors and early leukaemic precursors, ectopic expression of *scl* induces a proliferative stimulus under sub-optimal culture conditions via an anti-apoptotic effect, which are accompanied by a marked inhibitory effect on differentiation. They also demonstrated that the proliferative effect of *SCL* depends on the integrity of the cell cycle checkpoints of the host cell, as observed for *c-Myc* and other oncogenes, and requires both DNA-binding and the heterodimerisation domains.

Although *SCL* is oncogenic in T-cells, the *scl* transgenic mice do not show any alterations in T-cell development and do not develop tumours (Robb *et al.*, 1995b). Neither does the transgene enhance tumorigenesis induced by infection with the Moloney murine leukaemia virus. The *scl*-null mice die early in embryonic development and have no mature red blood cells (Robb *et al.*, 1995a; Shivdasani *et al.*, 1995). Neither erythroid nor myeloid progenitors can be detected in yolk sacs, the normal source of haemopoietic progenitors in the early embryo. Analysis of chimeric mice demonstrated that the *scl*-null ES cells make a substantial contribution to all non-haemopoietic tissues but do not contribute to any haemopoietic lineage (Porcher *et al.*, 1996; Robb *et al.*,

1996). *In vitro* differentiation assays showed that both primitive and definitive haemopoietic cells are absent in the *scl*-null embryonic bodies. Taken together, these results suggest that *SCL* functions very early in haemopoietic development, either in specification of ventral mesoderm to a blood cell fate, or in formation or maintenance of immature progenitors.

No target gene for *SCL* has been identified so far. Similar to *GATA-1*, the *SCL* activity also seems to be subject to the regulation by growth factors and differentiation inducers, and transcriptional control, phosphorylation and direct protein-protein interactions are three important mechanisms. Prasad *et al.* (1995) reported that Epo elicits a rapid, dose-dependent increase in the *scl* mRNA by increasing transcription of the gene and stabilising one of its transcripts. Furthermore, Epo also induces phosphorylation of the *SCL* protein, which results in an increase in DNA-binding activity. The full-length *SCL* protein binds a CANNTG element (E box) as a heterodimer with widely expressed *E12* and *E47*, both bHLH protein products of the *E2A* gene (Hsu *et al.*, 1991; Voronova and Lee, 1994). Such heterodimers may act by inhibiting the activation effects mediated by *E12* or *E47* (Doyle *et al.*, 1994). More interesting is the subsequent demonstration of a direct interaction between *SCL* and a second T-cell oncoprotein, the *LIM*-domain protein *LMO-2* (previously known as *Rbtn-2 or TTG-2*) (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). This interaction may be physiological relevant because *LMO-2* is co-expressed with *scl* in normal haemopoietic cells, and its "knockout" phenotype is remarkably similar to that of *scl* (Warren *et al.*, 1994). A final mechanism of the *SCL* regulation is mediated by another bHLH protein, *Id.* The *Id* proteins lack the basic region for transactivation but retain the oligomerisation domain; thus, they can counteract the activating function of bHLH systems via Id/bHLH interaction and impede formation of bHLH/bHLH homo- or heterodimers. The *Id* mRNA decreases in various cell lines when they are induced to differentiate (Benezra *et al.*, 1990), and over-expression of *Id* suppresses muscle, myeloid and erythroid differentiation (Jen *et al.*, 1992; Kreider *et al.*, 1992; Shoju *et al.*, 1994), suggesting that *Id* is a negative regulator of differentiation. Interestingly, Condorelli *et al.*, (1995) showed that the murine and human glutathione-S-transferase-*Id2* polypeptides compete with the *SCL/E2A*-specific DNA-binding activity when added to nuclear extracts derived from erythroid culture cells, suggesting a functionally-important interaction of *Id2* with the *SCL/E2A* complex.

Chapter 3: Erythroleukaemia

3.1. Leukaemia and general mechanisms of leukaemogenesis.

Leukaemia results from uncontrolled proliferation or expansion of haemopoietic cells that do not retain the capacity to differentiate normally into mature blood cells (reviewed by Sawyer *et al.*, 1991). The initiation and progression of leukaemia involve alterations in the normal homeostatic mechanisms used to regulate the self-renewal, proliferation, differentiation and apoptosis of blood cells. The study of leukaemogenic animal retroviruses, and the cloning of chromosomal translocation breakpoints from leukaemia patients, has led to identification of many proto-oncogenes involved in the development of leukaemia. These genes mainly encode growth factors, growth factor receptors, transcription factors and apoptotic proteins.

The first demonstration of the link between growth factors or their receptors and leukaemogenesis was the identification of the M-CSF receptor as the cellular counterpart of the *v-fms* oncogene of the Susan McDonough strain of feline sarcoma virus (Sherr *et al.*, 1985). The *c-fms* gene encodes a tyrosine kinase transmembrane receptor protein normally expressed in the monocyte-macrophage lineage that is dependent on its ligand M-CSF for activation of its kinase and growth-stimulatory properties. Its virally-encoded counterpart, *v-fms*, however, encodes a protein that has constitutive kinase activity. Mice reconstituted with v-fms-infected bone marrow develop malignancies of multiple haemopoietic lineages including erythroleukaemia, B-cell lymphoma and non-clonal myeloproliferative syndromes (Heard *et al.*, 1987). Sequencing of the *v-fms* gene identified a point mutation that results in the constitutive kinase activity and analogous mutations were also detected in about 10% of patients with acute myeloid leukaemia and myelodysplastic syndromes (Ridge *et al.*, 1990).

In addition to growth-regulating genes, genes involved in differentiation regulation, especially those encoding transcription factors, are also implicated in leukaemogenesis. As mentioned in Section 2.2.2, the T-cell leukaemia oncogene *scl* encodes a basic helix-loop-helix transcription factor and has been recently found to be essential for development of all haemopoietic lineages (Porcher *et al.*, 1996; Robb *et al.*, 1996). It is implicated in human T cell acute lymphoblastic leukaemia through its involvement in the t(1;14) chromosomal translocation and, more frequently, as a result of tumour-specific interstitial deletion on chromosome 1 (Finger *et al.*, 1989; Aplan *et al.*, 1990). The consequence of both these chromosomal alterations is over-expression of *scl* in leukaemic cells, which is believed to induce differentiation arrest of cells at an early stage.

Finally, dysregulation of apoptosis can also lead to leukaemia and this was demonstrated by the *bcl-2* gene and its role in human lymphomagenesis. This gene was first described in association with the t(14;18) chromosomal translocation, which is characteristically present in 85% of follicular lymphomas and results in the transcriptional activation and over-production of the *Bcl-2* protein (Tsujimoto *et al.*, 1985; also see Chapter 5). It proved to be a novel proto-oncogene that exerts its effect by inhibiting apoptosis in a cell population that normally is destined to die. This results in a secondary expansion of cells that, though they may not be themselves malignant, have an increased susceptibility to subsequent transformation events.

In sum, alteration of proto-oncogenes disrupts the normal development and maturation of haemopoietic cells regulated by growth factors and the associated stromal cells, and leads to uncontrolled growth factor- or stroma-independent growth and blockade in differentiation and apoptosis. Several experimental models indicate that co-operation between these oncogenes are required to generate a full leukaemic phenotype. However, not all molecular changes associated with leukaemia progression involve activation of oncogenes or inactivation of tumour supressor genes. Certain virus do not harbour cellular oncogenes but contain viral proteins that can stimulate host cell growth by acting as "pseudoligands" for cellular growth factor receptors, which results in growth factor-independent growth. A typical example is *gp55* of the *env* gene of spleen focus-forming virus (SFFV) that binds to and activates the Epo receptor, thereby inducing Epo-independent burst formation, confering Epo-independent growth to Epo-dependent cell lines, and promoting leukaemogenesis *in vivo* (Hankins *et al.*, 1978; Ruscetti *et al.*, 1990; Li *et al.*, 1990; also see Section 3.2)

3.2. Friend virus-induced erythroleukaemias.

Few of the murine retroviruses induce erythroleukaemia; most affect lymphoid cells or other myeloid lineages. However, the Friend virus (FV) specifically transforms erythroid progenitor cells (reviewed by Ben-David and Berstein, 1991). FVs are complexes of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent Friend murine leukaemia virus (F-MuLV). The presence of F-MuLV in the FV complexes is required to provide functions necessary for the replicative life cycle of SFFV. Although F-MuLV alone does not induce diseases in adult mice, it does induce spenomegaly, severe anaemia and erythroleukaemia when inoculated into new-born BALB/c or NIH/Swiss mice. The erythroleukaemia induced by the various strains of FV are multi-stage malignancies characterised by the polyclonal proliferation of non-leukaemic erythroid progenitor cells, followed by a later stage in which there is clonal or oligoclonal expansion of malignant cells. Thus, the evolution of Friend leukaemia embraces the general principles of leukaemogenesis described above. This section will

discuss the role of autocrine processes, the *p53* tumour suppressor gene and members of the *ets* gene family. In addition, host genes that control susceptibility to FV will also be discussed in the light of advances in our understanding of their mechanisms of action.

3.2.1. Early events.

The early stages of the diseases induced by FV are associated with a marked increase in the number of erythroid progenitors (BFU-E and CFU-E) shortly after virus inoculation. These infected cells are not immortal or transplantable, have limited self-renewal capacity, and retain the capacity for terminal differentiation to mature red blood cells. The polyclonal expansion of erythroid progenitor cells is due to the *env* gene of SFFV which encodes a 55kDa glycoprotein (*gp55*). As mentioned above (see Section 3.1), *gp55* binds to and activates the Epo receptor (Epo-R). By acting as a "pseudoligand" for Epo, *gp55* can induce Epo-independent burst formation, confer Epo-independent growth to Epo-dependent cell lines and promote leukaemogenesis *in vivo* (Hankins *et al.*, 1978; Ruscetti *et al.*, 1990; Li *et al.*, 1990). These studies explain the molecular basis for the early polyclonal stage of Friend leukaemia and the erythroid specificity of the proliferative stimulus induced by SFFV. The importance of Epo-R in Friend leukaemia is reinforced by two observations. First, several F-MuLV-induced erythroleukaemia cell lines have been isolated in which the Epo gene is rearranged and amplified and which constitutively produce Epo (McDonald *et al.*, 1987; Hankins *et al.*, 1989), or in which the Epo-R gene has been activated by proviral insertion (Lacombe *et al.*, 1991). Second, infection of mice with retroviral vectors carrying either the Epo gene or an activated Epo-R gene leads to erythroblastosis strongly reminiscent of the early stages of Friend disease (Hoatlin *et al.*, 1990).

3.2.2. Late events.

However, expression of *gp55*, and therefore activation of Epo-R and polyclonal expansion of erythroid progenitor cells, is not sufficient to induce a truly malignant disease. Only at a later time after infection (4-8 weeks) do malignant clones emerge that are capable of transplantation *in vivo* and growth in semisolid or liquid medium *in vitro*. This suggests that additional events are required for leukaemic transformation. These events include both inactivation of the *p53* tumour suppressor gene and activation of oncogenes that belong to the *ets* family of oncogenes. It was reported that approximately 30% of leukaemic clones transformed by FV have an inactivated *p53* gene as the result of internal deletions or SFFV proviral insertions (reviewed by Lane and Benchimol, 1990). Many of these clones no longer retain a wild-type *p53* gene, indicating that reduction to homozygosity frequently accompanies such inactivation events. The importance of *p53* in the evolution of Friend leukaemia is also evident from studies on

transgenic mice that express high levels of mutant forms of the *p53* protein. In addition to displaying an increased spontaneous predisposition to a variety of malignancies, these *p53* transgenic mice are more susceptible to the induction of the late stages of the leukaemia induced by FV (Lavigueru and Bernstein, 1991). Thus, inactivation of the *p53* gene, either somatically after infection with FV or as the result of transgenic inheritance of dominant-negative alleles of *p53*, appears to be a central event in the emergence of the late stages of this leukaemia. These observations also imply that the accumulation of a specific set of mutational events, rather than the order in which they occur, is important for leukaemia induction.

In addition to inactivation of the *p53* gene, a cellular proto-oncogene, *spi-1* (SFFV proviral integration 1), is activated through proviral insertion in 95% of erythroleukaemia induced by FV but not in other murine leukaemias (Moreau-Gachelin et al., 1988). The high frequency of *spi-1* rearrangements in SFFV-induced leukaemic clones strongly implies that this locus is essential for transformation of erythroid cells during the progression of Friend disease. *Spi-1* transcripts are highly expressed in the cell lines in which the *spi-1* locus is rearranged (Moreau-Gachelin *et al.*, 1990). *Spi-1* proved to encode a DNA-binding transcription activator that belongs to the *ets* oncogene family (Klemsz *et al.*, 1990; the *ets* oncogene family is discussed in detail in Chapter 4). However, *spi-1* is not rearranged or activated in the leukaemia induced by F-MuLV (Moreau-Gachelin *et al.*, 1988). Instead, there is another highly preferred integration site for F-MuLV, termed *fli-1* (Friend leukaemia integration 1), in over 75% of erythroleukaemia induced in either BALB/c or NIH/Swiss mice inoculated at birth with F-MuLV. Other haemopoietic neoplasms induced by F-MuLV, including myeloid and lymphoid tumours, do not show rearrangement of the *fli-1* locus, nor have any of the 35 erythroleukaemia cell lines induced by FV. Interestingly, *fli-1* encodes another member of the *ets* family transcription factors (Ben-David *et al.*, 1991; discussed in detail in Section 4.2). Taken together, these experiments suggest that activation of *fli-1* or *spi-1* and inactivation of *p53* are highly frequent and hence important steps in the evolution of Friend erythroleukaemia.

3.2.3. Three host genes - *W*, *Sl*, and *Fv-2* - that control susceptibility to Friend virus.

At least three host genes specifically control susceptibility to leukaemia induction by FV. Two of these genes, *W* and *Sl*, were first identified on the basis of pleiotropic developmental defects: mice carrying *W* or *Sl* mutations exhibit a dominant "white spotting" phenotype, anaemia and sterility. Earlier transplantation experiments suggest that the *W* locus controls an intrinsic property of the stem cells that give rise to melanocyte, erythrocytes and germ cells; while the *Sl* locus affects the microenvironment in which these stem cells develop during embryogenesis and function

in adult life. Consistent with these conclusions, subsequent results have shown that *W* encodes the *c-kit* receptor tyrosine kinase, while *Sl* encodes the ligand for this transmembrane receptor, stem cell factor (SCF) (reviewed by Berstein *et al.*, 1991). In the light of these insights into the molecular basis of the *W* and *Sl* phenotypes, it is interesting to speculate on the mechanisms of resistance of *W* and *Sl* mice to FV. Both *W* and *Sl* mutant mice have reduced numbers of erythroid progenitors, the likely cellular targets for transformation by FV, and thus resistance may reflect a reduction in the number of target cells. Alternatively, activation of the *c-Kit* signalling pathway may be a necessary event in erythroleukaemia induction.

Like the *W* locus, *Fv-2* also determines a cell-autonomous, intrinsic property of the target cells, not their environment (Silver and Teich, 1981). By analysing strains of mice congenic for *Fv-2*, Del Rizzo *et al.* (1988) have shown that the majority of BFU-E erythroid progenitors from $Fv\text{-}2^{rr}$ mice are not actively cycling, whereas a high proportion of BFU-E from $Fv\text{-}2^{ss}$ mice are. This difference in cell cycling status between $Fv\text{-}2^{rr}$ and $Fv\text{-}2^{ss}$ congenic mice is restricted to the BFU-E stage of erythroid differentiation. These observations are reminiscent of earlier experiments showing that cell cycling is required for successful execution of early steps in the retroviral life cycle, and suggest that *Fv-2* may encode a novel cell type-specific regulator of the mammalian cell cycle.

CHAPTER 4: The *Ets* Family of Proteins: Transcription Factors and Oncoproteins

The *v-ets* oncogene, the first member of the *ets* gene family, was discovered as part of a fusion protein with *gag* and *myb* expressed by the E26 avian erythroblastosis virus, from which it derives its name (E26 transformation-specific). Since then, more than 30 related genes have been discovered and cloned from a variety of organisms ranging from humans to Drosophila, and these include *c-ets-1*, *c-ets-2*, *elf-1*, *elf-2*, *elk-1*, *erg*, *E1AF*, *E4TF1*, *ERF*, *ERM*, *fli-1*, *PEA3*, *SAP-1*, *spi-1/PU.1*, *tel*. In most family members, the conserved DNA-binding domain (the Ets domain) is located at the carboxyl terminus of the protein, with the exception of *Elf-1*, *Elk-1* and *SAP-1*, where it is found at the amino terminus. The Ets domain, which covers approximately 85 amino acids has no structural homology to other known DNA-binding motifs, such as the zinc finger, homeodomain, leucine zipper or helix-turn-helix motifs, but has limited homology to the DNA-binding domain of the *c-myb* oncogene product. This chapter will first discuss the general features of the *Ets* protein family (reviewed by Crepieux *et al.*, 1994; Hromas and Klemsz, 1994; Wasylyk *et al.*, 1993; MacLeod *et al.*, 1992; Seth *et al.*, 1992), followed by a more detailed description of those that are directly relevant to the work presented by this thesis (i.e. *Fli-1* and *Erg*).

4.1. General features of the *Ets* family of proteins.

4.1.1. Tissue distribution.

The *Ets* proteins are expressed in a wide variety of tissues and the pattern of tissue-specific expression varies among different family members. For example, *c-ets-1* is expressed preferentially in adult lymphoid tissue, with high levels in the thymus, and at lower levels in the lung and spleen. In contrast, *c-ets-2* is expressed in almost every type of tissues and cell lines tested. *Elk-1* is expressed only in the lung and testis. *Spi-1/PU.1* is found to be expressed primarily in the B-cells and myeloid cells.

4.1.2. The *Ets* proteins as transcription factors.

DNA-binding activity. The *Ets* proteins have been found to bind specifically to sequences containing an invariant core motif GGAA/T in the middle of 10bp of DNA. The flanking sequences are variable and there is growing evidence that they help determine which *Ets* protein will bind. The DNA-binding activity of the *Ets* proteins is mediated by the conserved Ets domain which, on its own, appears to be sufficient for specific DNA-binding. Nuclear magnetic resonance (NMR) structural analyses have

determined that the Ets domain displays a "winged helix-turn-helix motif" (Donaldson *et al.*, 1996), demonstrating that the Ets domain is compact and highly well folded. Nevertheless, this minimal DNA-binding domain is modulated by the remainder of the protein (see below). ***Intramolecular inhibitory domains.*** The activity of some of the *Ets* proteins as transcription factors is negatively regulated by an intramolecular mechanism. Studies of deletion mutants and natural variants of *c-Ets-1* (Lim *et al.*, 1992) indicate that the flanking regions negatively regulate the *Ets* domain, since N-terminal deletion mutants display higher binding activity than the full-length protein, so as deletions of residues C-terminal to the *Ets* domain. These studies led to a model of two inhibitory regions working together to sterically mask the DNA-binding surface. These inhibitory domains can be relieved by association with accessory factors. ***Transactivation domains.*** Expression of the *Ets* proteins activates transcription from multimerised DNA motifs, suggesting that they contain activation domains. The transactivation domains reside in non-conserved regions and act independently from DNA-binding domains. *c-Ets-1* and *c-Ets-2* fused to heterologous DNA-binding domains are able activate through the corresponding heterologous motifs (MacLeod *et al.*, 1992). ***The Ets proteins as components of the general transcription machinery.*** In addition to the role of *Ets* family members as classical activators that stimulate transcription from a distance, they may also have a primary role in the formation of transcription initiation complex on minimal core promoters containing or lacking the TATA sequence. It has been reported that recombinant *Spi-1/PU.1* can bind to TFIID (Hagemeier *et al.*, 1993), the TATA-box binding protein that stimulates RNA polymerase II to initiate transcription. Moreover, some ets motifs are located close to the transcription initiation site in a number of promoters that lack a TATA element, and these have been shown to be important for transcription. It is possible that the *Ets* proteins may be a component of the "general transcription complex" that forms on certain promoters around the start site. This may give them a primary switching role during differentiation or in phases of the cell cycle.

The *Ets* family members regulate a wide spectrum of target genes in response to different cellular signalling pathways (see Section 4.1.4). This ability to mediate diverse cellular processes is achieved, at least in part, through co-operation with other groups of transcription factors. Co-operation can affect DNA-binding or transactivation activity, which may result in transcriptional activation or repression. ***Co-factors that physically interact with the Ets proteins.*** A number of transcription factors have been shown to physically interact with the *Ets* proteins and form ternary or quaternary complexes at the recognition sites. These include serum response factor (SRF), the *c-Fos/c-Jun* complex, *NF-EM5*, *Pax-5*, retinoblastoma binding factor 1 (*RBF-1*), *MafB*, retinoblastoma protein (*Rb*), *Rbtn-2*. Among those are SRF and the *c-Fos/c-Jun* complex that have attracted the most attention. SRF binds to a motif adjacent to the *ets*-like motif. *Elk-1* (Hipskind *et al.*, 1991), *SAP1* (Dalton and Treisman, 1992) and *Fli-1* (Watson *et al.*, 1997) can

recognise and interact with the SRF bound to the DNA, and form a stable ternary complex. In some cases, the *c-Fos/c-Jun* complex also requires co-operation with certain Ets proteins, such as *c-Ets-1* (Bassuk and Leiden, 1995), *c-Ets-2* (Wu *et al.*, 1994), *Elf-1* (Wang *et al.*, 1994) and *Erg* (Buttice *et al.*, 1996), via direct protein-protein interactions for transactivation. In the case of transactivation of the polyomavirus enhancer by *c-Ets-1* and the *c-Fos/c-Jun* complex, cooperativity is not mediated at the level of DNA binding (Wasylyk *et al.*, 1990). Direct interactions with some transcription factors can also lead to repression of the *Ets* protein activity. *MafB*, an *AP-1* like protein, binds to the DNA-binding domain of *c-Ets-1* via its basic region or leucine-zipper domain (Sieweke *et al.*, 1996), and this represses the ability of *c-Ets-1* to transactivate either a synthetic promoter containing *Ets*-binding sites or the promoter of transferrin receptor. In addition, unphosphorylated *Rb* can also reduce the transcriptional stimulating activity of *Elf-1* in inactive lymphocytes by direct physical conatct. Upon T-cell activation, Rb is phosphorylated and subsequently dissociates from the complex, which restores the *Elf-1* activity immediately (Wang *et al.*, 1993). Finally, a novel *Ets* family member, *Elf-2*, has been recently reported to bind to *Rbtn-2*, a T-cell oncoprotein that belongs to the *LIM* family of transcription factors, though the significance of this interaction is unclear (Wilkinson *et al.*, 1997). ***Other co-factors.*** The *Ets* proteins can also cooperate with a number of other factors, such as *c-Myb*, *C/EBP*α. However, the mode of these interactions have not been established. For example, *c-Ets-2* does not activate the *mim-1* promoter unless *c-Myb* is also present (Dudek *et al.*, 1992). Furthermore, *c-Myb* can synergise with either *c-Ets-1* or *c-Ets-2* to transactivate the promoter of an early myeloid gene (Shapiro, 1995). The nature of such co-operation is not known and a direct interaction between *c-Myb* and *c-Ets-1* or *c-Ets-2* has not been documented. Finally, the *Ets* family member also seem to co-operate with one another in gene activation. Hume *et al.* (1997) reported that *Spi-1/PU.1* and *c-Ets-2* can synergistically transactivate the *c-fms* promoter. Moreover, *ERF* has been identified as an *Ets* repressor factor that exhibits strong transcriptional repressor activity on promoters that contain an *Ets*-binding site (Sgouras *et al.*, 1995).

4.1.3. The *Ets* proteins as oncoproteins.

The v-Myb-Ets fusion protein in E26 induces erythroblastosis. Various lines of evidence show that *v-Ets* is responsible for the erythroblastosis induced by the avian acutely transforming retrovirus, E26. E26, which expresses a *v-Myb-Ets* fusion protein, causes a mixed erythroid and myeloid leukaemia with a predominance of the former, whereas AMV, that expresses only *v-Myb*, induces myeloid leukaemia. The temperature-sensitive mutant of E26, ts1.1E26, with a point mutation in the *v-Ets* DNA-binding domain, is temperature-sensitive *in vitro* for erythroid but not myeloid transformation.

Activation by proviral insertion. Two different *ets* genes, *fli-1* and *spi-1/PU.1*, are activated by proviral insertion, and the activation is found to be responsible for virally induced murine erythroleukaemia (also see Section 3.2.2, 4.2 and 4.3). Proviral integration by the Friend murine leukaemia virus (F-MuLV) and the spleen focus-forming virus (SFFV) into flanking sequences activates expression of the *fli-1* and *spi-1/PU.1* genes, respectively.

Activation by chromosomal translocation. Chromosomal translocations involving the *ets* oncogenes have been associated with both haemotological and solid tumours. In many cases, chimeric proteins are created as a result of fusion between the two genes located at the sites of the breakpoint. For example, the human *c-ets-1* and *c-ets-2* genes are translocated in certain types of acute leukaemia from chromosomes 11 to 4 and from 21 to 8, respectively. Approximately 85% and 10% of the human Ewing's sarcomas and the related tumours are recurrently associated with t(11;22) and t(21;22) chromosomal translocations which result in the production of aberrant fusion proteins *EWS/Fli-1* and *EWS/Erg*, respectively (Delattre *et al.*, 1992; Sorensen *et al.*, 1994). The fusion proteins consist of the amino terminus of *EWS*, a putative RNA-binding protein, fused to the carboxyl terminus of *Fli-1* or *Erg* including the *Ets* domain. *EWS/Fli-1* and *EWS/Erg* also display the biochemical attributes of a transcription factor. Accumulating evidence suggests that *EWS/Fli* and *EWS/Erg* may disrupt normal growth and differentiation by acting as aberrant transcription factors that inappropriately activate and/or repress the expression of target genes. This may also underlie the mechanism whereby another fusion protein of two transcription factors, the *Ets* protein *Tel* and *AML1*, contributes to paediatric acute lymphoblastic leukaemia (ALL) (Shurtleff *et al.*, 1995). A second chromosomal translocation in ALL, t(9;12), fuses *Tel* to a tyrosine kinase, *Abl* (Papadopoulos *et al.*, 1995). In addition, *Tel* is also involved in the t(5;12) translocation associated with chronic myelomonocytic leukaemia (CMML), which results in the amino terminus of *Tel* being coupled to the tyrosine kinase domain of PDGF (Golub *et al.*, 1994; Carroll *et al.*, 1996). Both *Tel/Abl* and *Tel*/PDGF fusion proteins have an elevated tyrosine kinase activity, which is thought to be the consequence of constitutive dimerisation that is mediated by the helix-loop-helix domain of *Tel* that is retained in the fusion proteins.

In vitro transformation by the Ets proteins. The role of *ets* gene family members as oncogenes has been tested in classical transformation experiments in which over-expression of the *Ets* genes, such as *c-ets-1*, *c-ets-2*, *erg* or *fli-1*, in NIH-3T3 mouse fibroblasts generates transformed foci in low serum conditions. These transformed cells grow in soft agar and form tumours in nude mice (Wasylyk *et al.*, 1993).

4.1.4. The functions of *Ets* proteins in development and co-ordinated regulation of cell proliferation, differentiation and apoptosis.

Attempting to assess the biological functions of *Ets* proteins from the identity of their target genes is a difficult quest, given that many members appear to be expressed in an array of tissues, where they may regulate common or specific target genes. To date, a large number of *Ets* protein target genes have been determined (see Table 4.1). However, caution must be exercised with regard to defining genuine target genes as, in many cases, only putative *Ets*-binding sites are identified in the promoters of genes of interest; furthermore, *in vitro* transactivation activity does not always correlate with an *in vivo* function. As can be seen in Table 4.1, the *Ets* protein target genes can be classified into six categories: growth factor and growth factor receptors, oncogenes and tumour suppressor genes, genes involved in or regulating extracellular matrix (ECM) interactions (e.g. integrins and metalloproteinases), lymphoid-specific genes, viral enhancers, and genes encoding cytoskeleton components. This suggests that the *Ets* proteins may play a role in co-ordinated regulation of growth and differentiation in response to growth factors and differentiation inducers, in lymphoid development and functions, and in viral infectious cycles. Additionally, the involvement of *Ets* proteins in the transcription of metalloprotease genes should be emphasised. Metalloprotease genes are involved in the degradation of ECM and many of them, such as those encoding stromelysin 1, collagenase and urokinase plasminogen activator (μPA), contain *Ets*-binding sites in their regulatory flanking sequences. Furthermore, *c-Ets-1* (Wasylyk *et al.*, 1993; Buttice and Kurkinen, 1993; Nerlov *et al.*, 1992), *Erg* (Buttice *et al.*, 1996) and *E1AF* (Kaya *et al.*, 1996) are able to transactivate the promoters of those genes *in vitro*. Moreover, transfection of the non-invasive human breast cancer cell line with the *E1AF* expression plasmid results in induction of invasive and motile activities, accompanied by an increase in the type IV collagenase (MMP9) gene expression (Kaya *et al.*, 1996). Tumours derived from the *E1AF* transfectants are highly invasive and produce MMP-9. Elevated expression of *E1AF* and MMP-9 were also observed in several invasive tumour cell lines. Take together, this suggests that the *Ets* proteins play a role in co-ordinating events that lead to the degradation of ECM, an important process in vasculogenesis, angiogenesis, and tumour cell growth, invasion and metastasis.

Clues with regard to the functions of *Ets* proteins are also obtained in situations where the *ets* gene expression is manipulated, either in cell lines *in vitro* or in the entire organisms *in vivo*. Over-expression of *spi-1/PU.1* in Friend virus-induced murine erythroleukaemia (MEL) cells leads to differentiation inhibition and, as a result, these cells can no longer induce β-globin expression in response to DMSO (Yamada *et al.*, 1997). Intriguingly, over-expression of *spi-1/PU.1* also inhibits growth and, in conjunction with DMSO treatment, induces apoptotic cell death. Although it is

Table 4.1. List of genes whose promoters contain putative *Ets*-binding sites and/or can be transactivated by the *Ets* proteins (in italic).

Growth factors and growth factor receptors *GM-CSF* G-CSF IL-2, -3, -4 and -6 NGF α subunit *GM-CSF receptor α* *CSF-1 (or M-CSF) receptor (c-fms)* *Thrombopoietin receptor (MPL)* Vascular endothelial growth factor receptor (Flt-1) *Transferrin receptor* *Macrophage scavenger receptor*	**Oncogenes and tumour suppressor genes** *c-ets-1* *c-ets-2* *c-fos* *c-myc* c-myb *JunB* *spi-1/PU.1* p53 Rb
Genes involved in or regulating extracellular matrix (ECM) interactions integrin CD11c integrin CD18 integrin α4 *collagenases* *stromelysin 1* *urokinase plasminogen activator (μPA)*	**Genes encoding cytoskeleton proteins** vimentin *Endo A/B (type II/I karetin)*
Lymphoid-specific genes T-cell receptor α chain *T-cell receptor β chain* IgH Igκ MHC class I H2-K gene MHC class II gene I-A3 *CD4*	**Viral enhancers** *Adenovirus E1A enhancer* *EBV early cytoplasmic antigen* *HIV-1 LTR* *HTLV-1 LTR* *MSV LTR* *Polyoma enhancer* *SV40*
Others *GATA-1* CD34 (a stem cell antigen) *Mim-1*	

puzzlying how *Spi-1/PU.1* can inhibit growth and differentiation at the same time, and how a protein that inhibits growth and induces apoptosis can induce erythroleukaemia, the results suggest a role of *Spi-1/PU.1* in co-ordinated regulation of cell growth, differentiation and apoptosis. Consistent with this theme, but contradictory to the data, are the studies by Delgado *et al.* (1994) who demonstrated that inhibition of the *spi-1/PU.1* expression in MEL cells by antisense oligonucleotides results in a significant decrease in proliferation and cloning efficiency of those cells. Furthermore, this anti-proliferative effect is not related to an apparent maturation of the erythroleukaemic cells. Targeted disruption of the normal *spi-1/PU.1* gene *in vivo* also suggests that it is required in the development of multiple haemopoietic lineages and, therefore, it may act at the level of multipotential progenitors (Scott *et al.*, 1994; McKercher *et al.*, 1996). In contrast, targeted inactivation of *c-ets-1* by the *RAG-2*$^{-/-}$ complementation system and murine embryonic stem (ES) cells containing homozygous deletion of the *c-ets-1* gene (*ets-1*$^{-/-}$) demonstrated that the functions of *c-Ets-1* are restricted to lymphoid development and functions (Bories *et al.*, 1995; Muthusamy *et al.*, 1995). *Ets-1*$^{-/-}$*-RAG-2*$^{-/-}$ chimaeric mice display markedly decreased numbers of mature thymocytes and peripheral T cells, which show a severe proliferative defect in response to multiple activation signals and demonstrate increased rates of spontaneous apoptosis *in vitro*. In contrast, B-cells are present in normal number in the *ets-1*$^{-/-}$ mice, but a large proportion are IgM plasma cells. These findings suggest that *c-Ets-1* is essential for the maintenance of the normal pool of resting T- and B-lineage cells.

4.1.5. Regulation of the *Ets* functional activity.

A variety of growth modulators and differentiation inducers affect the *Ets* activity, and this regulation is controlled mainly at three levels: the initiation of transcription, post-translational modification and protein stability.

Transcriptional regulation. It has been reported that the *c-ets-1* and *c-ets-2* gene expression is modulated at the transcriptional level in response to growth factors and during T-cell activation. Gilles *et al.* (1996) demonstrated that *c-ets-1* is an early-response gene of TNFα and IL-1α, since it undergoes a rapid (within 1h) and long-lasting (19h) increase upon stimulation with these cytokines. In contrast, bFGF, EGF and PDGF are mainly delayed stimulators. In T-cells, the expression of *c-ets-1* is highest in quiescent cells and decreases following T-cell activation (Bhat *et al.*, 1990). Conversely, the *c-ets-2* expression increases from a low level following T-cell activation and reaches the maximum level 1-2h after induction. The mechanism of co-ordination of the expression of these genes is unclear.

Phosphorylation. Various growth factors and differentiation inducers modulate *Ets* protein activity by eliciting a series of signalling pathways, and one of the most important consequences is phosphorylation of the proteins which can lead to a change in DNA-binding or transactivation activity. Alternatively, phosphorylation appears necessary for the recruitment of co-activators in order to transactivate efficiently, as is the case for *Spi-1/PU.1* (Pongubala *et al.*, 1993). Numerous studies suggest that various *Ets* proteins are direct targets of the *Ras/Raf*/MAP kinase signalling pathway. Expression of *Ha-Ras* can induce phosphorylation of *c-Ets-1* (Yang *et al.*, 1996; Wasylyk *et al.*, 1997), *c-Ets-2* (Yang *et al.*, 1996; Wasylyk *et al.*, 1997), *Elk-1* (Marais *et al.*, 1993), *ERM* (Janknecht *et al.*, 1996) and *PEA3* (Ohagan *et al.*, 1996) by activating MAP kinase family proteins, which subsequently strongly activates their transactivation activity. Interestingly, *Elk-1*, in turn, stimulates MAP kinase autophosphorylation and activity (Rao and Reddy, 1993b), and this is mediated by direct interaction between the two proteins (Rao and Reddy, 1994). Consistent with the role of *Ras* signalling pathway in regulating *Ets* family members is the fact that several dominant negative mutants of the *Ets* proteins (*c-Ets-1*, *c-Ets-2* and *Spi-1/PU.1*) can revert the transformed phenotypes of NIH3T3 fibroblasts due to *ras* over-expression, including morphology, anchorage independent growth, saturation density, growth in low serum and tumour formation in nude mice (Wasylyk *et al.*, 1994). In addition to the MAP kinase family, the *Ets* proteins can also be phosphorylated by PKC (*Erg* and *Spi-1/PU.1*), PKA (*ERM*), *cdc2/cyclin B* kinase (*ERF*) and possibly Ca^{2+}-dependent calmodulin kinase (*c-Ets-1*, *c-Ets-2* and *Elf-1*). It is worthwhile mentioning that phosphorylation can also negatively regulate the functional activities of *Ets* proteins. Phosphorylation of *c-Ets-1* can inhibit its DNA-binding activity and its ability to restore mitogenic response of a cell line expressing a mitogenically defective CSF-1 receptor (Rabault *et al.*, 1996). As mentioned above, phosphorylation of *c-Ets-1* can also enhance its transactivation activity. Therefore, the consequence of *c-Ets-1* phosphorylation, in terms of activation or repression, may be dictated by biological and cellular contexts. Finally, phosphorylation of *ERF*, an *Ets* repressor factor, can also decreases its transcriptional repressor activity (Sgouras *et al.*, 1995).

Protein stability. Finally, protein stability is another level at which the *Ets* family members are regulated. The half-life of *c-Ets-2* is increased from 20min to 140min following phorbol ester stimulation of protein kinase C (Fujiwara *et al.*, 1988). The PEST sequence (regions rich in Pro, Glu, Ser and Thr) are found in many *Ets* proteins, such as *Erg, c-Ets-1, c-Ets-2, Fli-1* and *Spi-1/PU.1*, and may play a role in protein turnover. The PEST sequences are located between putative transactivation domains and DNA-binding domains and cleavage may create a DNA-binding domain free from the regulatory restraints imposed by amino-terminal domains.

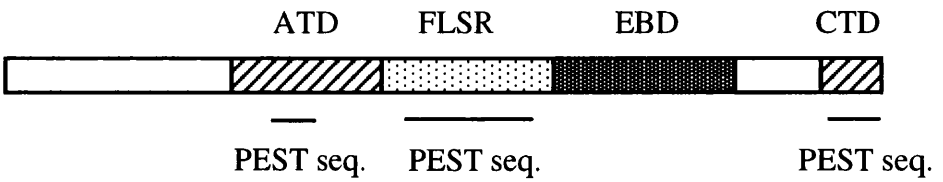
4.2. *Fli-1.*

The mouse *fli-1* gene was originally identified as the proto-oncogene insertionally activated in 75% of erythroleukaemia cell clones induced by Friend murine leukaemic virus (see Section 3.2.2 and 4.1.3). The human homologue of *fli-1* is rearranged in Ewing's sarcoma (see Section 4.1.3) and neuroepithelioma as a result of reciprocal chromosomal translocation. Expression of the *fli-1* transcripts is present at high levels in spleen and lymphoid tissues, such as thymus, and is also detectable in ovary, bone marrow and heart tissue (Nunn *et al.*, 1983; Watson *et al.*, 1992). In haemopoeitic cell lines, *fli-1* is expressed in a number of T-cells (CEM, Molt-4, Jurkat), B-cells (P3HR1) and erythroleukaemic cells (HEL, JK-1, TMK) (Nunn *et al.*, 1983; Watson *et al.*, 1992; Klemsz *et al.*, 1993). No expression of the *fli-1* mRNA is detectable in two of the erythroleukaemia cell lines, K562 or RM10 (Klemsz *et al.*, 1993).

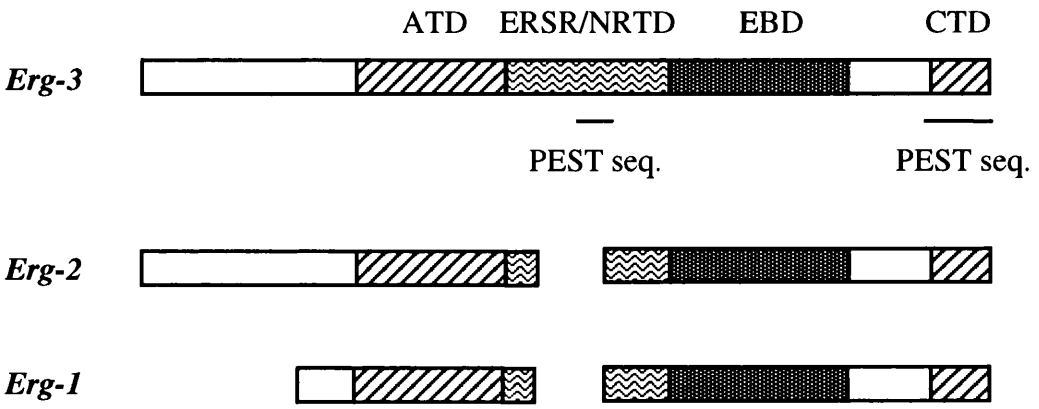
Fli-1 is a sequence-specific transcriptional activator by virtue of its ability to activate the transcription of a reporter gene that is linked to the *Fli-1* target sequences. Like other *Ets* proteins, DNA-binding of *Fli-1* is mediated by the conserved Ets domain (Figure 4.1a). Deletion analysis revealed the presence of two antonomous transcriptional activation domains, one at the amino-terminal region (amino-terminal transcriptional activation or ATA), and the other at the carboxy-terminal region (carboxy-terminal transcriptional activation domain or CTA) (Rao *et al.*, 1993a; Zhang *et al.*, 1993). The *Fli-1*-specific region (FLSR) and the conserved Ets domain are between these two transactivation domains. Interestingly, PEST sequences are found in these two transactivation domains, which may be important in regulating functional activity of the *Fli-1* proteins (Rao *et al.*, 1993). Recently, both *Fli-1* and *EWS/Fli-1* are found to recognise serum response element (SRE) and form a ternary complex with a second protein, serum response factor (SRF), in the promoters of *c-fos* and *egr1* (Magnaghi-Jaulin *et al.*, 1996; Watson *et al.*, 1997). Very few of the *Fli-1* target genes have been identified although DNA-binding sites for *Fli-1* have been identified in a number of lymphoid cell-specific genes. However, it has been reported that the human *Fli-1* protein can transactivate the HIV LTR core enhancer and the *gata-1* promoter (Hodge *et al.*, 1996; Seth *et al.*, 1993), the latter being consistent with a role of *Fli-1* in the regulation of erythropoeisis. Recent reports by Athanasiou *et al.* (1996) and Deveaus *et al.* (1996) further extend the functions of *Fli-1* to a role in controlling differentiation and gene expression along the megakaryocyte/platelet pathway. Athanasiou *et al.* (1996) discovered an elevated expression of *fli-1* in K562 cells induced to undergo megakaryocytic differentiation by phorbol ester. Moreover, over-expression of *fli-1* in those cells caused a higher level of spontaneous differentiation along megakaryocytic and erythroid lineages. Furthermore, Deveaus *et al.* (1996) demonstrated that *Fli-1* can

Figure 4.1. Schematic representation of the functional domains of the *Fli-1* (a) and *Erg* (b) proteins.

(a) *Fli-1*



(b) *Erg-1*, *Erg-2* and *Erg-3*



ATD: Amino-terminal transactivation domain
CTD: Carboxy-terminal transactivation domain
EBD: *Ets*-binding domain
PEST seq.: sequences rich in proline, glutamine, serine and threonine that are important in the regulation of protein stability
FLSR: *Fli-1*-specific region
ERSR: *Erg*-specific region
NRTD: Negative regulatory transactivation domain

transactivate the promoter of thrombopoietin receptor, whose ligand specifically controls megakaryocytic differentiation.

Consistent with the high levels of *fli-1* expression in the thymus and spleen, two recent reports from Dr. A. Bernstein's laboratory using a transgenic approach further confirm that *Fli-1* plays a vital role in lymphoid development and the associated immune functions, and the induction of erythroleukaemia by Friend virus. The first report detected a defect in thymus development and a delay in Friend virus-induced erythroleukaemia in mice that expressed a truncated mutant *Fli-1* protein (Melet *et al.*, 1996). The second report demonstrated that the *fli-1* transgenic mice develop a high incidence of a progressive immunological renal disease, and ultimately died of renal failure caused by tubulointerstitial nephritis and immune-complex glomerulonephritis (Zhang *et al.*, 1995). The hypergamma-glubulinemia, splenomegaly, B-cell hyperplasia, accumulation of abnormal $CD3^+B220^+$ T lymphoid cells and $CD5^+b220^+$ B-cells in peripheral lymphoid tissues, and detection of various autoantibodies in the sera of diseased *fli-1* transgenic mice suggest the involvement of an immune dysfunction in the pathogenesis of the renal disease. In addition, splenic B-cells from the transgenic mice exhibit increased proliferation and prolonged survival *in vitro* in response to mitogens, suggesting that programmed cell death is perturbed. Interestingly, transgenic mice overexpressing *bcl-2*, an anti-apoptosis gene (see Chapter 5), in B-cells also exhibit a similar enhanced antibody response and prolonged survival *in vitro*, and they develop a systemic autoimmune disease *in vivo* (Strasser *et al.*, 1991). These similarities between the *fli-1* and *bcl-2* transgenic mice indicate that these two proteins may have similar functional end points. It is possible that they act through distinct pathways which converge at some point. Alternatively, they may be part of the same pathway and, in this scenario, it is possible that *Fli-1* may lie upstream of *Bcl-2* and regulate its activity, directly or indirectly.

4.3. *Erg*.

The *erg* gene was identified by screening a cDNA library of human Colo-32 cells (a colon tumour-derived cell line) with a human *c-ets-2* cDNA (Rao *et al.*, 1987; Reddy *et al.*, 1987), and it was subsequently found to be involved in chromosomal translocations associated with human Ewing's sarcoma (Sorensen *et al.*, 1994; also see Section 4.1.3) and myeloid leukaemia (Shimizu *et al.*, 1993). In parallel with the involvement of *erg* in tumorigenesis, it proves to be a proto-oncogene capable of transforming NIH-3T3 mouse fibroblast cells *in vitro* (Hart *et al.*, 1995). It codes for three proteins, *Erg-1*, *Erg-2* and *Erg-3*, as a result of differential splicing, alternative polyadenylation and also alternative usage of the initiation codon (Rao *et al.*, 1987; Reddy *et al.*, 1987; Prasad *et al.*, 1994). The *erg* gene is expressed at high levels in transformed cells, and its

transcripts of ~3.2-3.6kb and ~5kb can be detected in various cell lines, such as Colo-32, KG-1 and Molt-4 (Rao *et al.*, 1987; Reddy *et al.*, 1987; Prasad *et al.*, 1994).

All *Erg* proteins proved to be sequence-specific transcriptional activators (Reddy and Rao, 1991; Prasad *et al.*, 1987). Structural analysis of the *Erg* proteins (Siddique *et al.*, 1993) indicates that, like *Fli-1*, they also have two transactivation domains (ATD and CTD) residing at the amino- and carboxyl-terminus of the proteins (Figure 4.1b). Between ATD and CTD are the *Erg*-specific region (ERSR) and the Ets domain. It is worthwhile noting that, overall, the human *Erg* and *Fli* proteins show 80% homology throughout the coding regions, whereas within the 84-amino acid Ets domain there is 98% sequence identity (Ben-David *et al.*, 1991). As the *Ets* domain is responsible for sequence-specific DNA-binding activity, it is likely that *Erg* and *Fli-1* have similar sequence specificity. On the other hand, the ERSR of *Erg* proteins shows no homology to FLSR of *Fli-1*, suggesting that this region may dictate the functional differences between *Erg* and *Fli-1* (Prasad *et al.*, 1994). The downstream cellular genes regulated by *Erg* are also poorly documented. However, it has been reported recently that *Erg* can transactivate the human collagenase 1 promoter when recruited by *c-Fos/c-Jun* at the promoter (Buttice *et al.*, 1996). Collagenase 1 belongs to a family of matrix-metalloproteinase (MMPs) that are extracellular enzymes, which degrade connective tissues and basement membranes and are expressed widely during growth and development. In mature adults, however, MMPs are rarely expressed except in places of diseases or active tissue-renewal/remodelling. For example, many MMPs are expressed in human tumours and are thought to play a critical role in several stages of tumour progression: tumour growth, tissue invasion and metastasis. Therefore, the fact that the *Erg* proteins regulate the collagenase 1 expression provides intriguing possibilities with regard to the molecular mechanisms of oncogene action. Like many other *Ets* proteins, *Erg* proteins are phosphoproteins. Phosphorylation of *Erg-2* can be stimulated by phorbol ester or activation of protein kinase C (PKC) but not by Ca^{2+} ionophore treatment (Murakami *et al.*, 1993), which, in turn, may modulate the functional activity of the protein.

CHAPTER 5: *Bcl-2* and Other Apoptosis Proteins

5.1. Programmed cell death and apoptosis.

Programmed cell death is an inconspicuous yet prevalent phenomenon in complex multi-cellular organisms, by which single cells are deleted from the midst of living tissues. The decision to die is made cell-autonomously and the cell is an active participant in its own death. With few exceptions, cellular death is accomplished by a process with a structural stereotype, termed apoptosis, that strongly suggests a common underlying mechanism. Apoptosis is regulatable and is of fundamental importance to tissue development and homeostasis. Dysregulated apoptosis is important in the pathogenesis of several important human diseases including neoplasia, and recognition of the defects involved is prompting development of new therapeutic strategies. The consistent morphological, cellular and molecular changes accompanying apoptosis include plasma membrane blebbing, cytoplasmic vacuolisation, chromatin condensation, DNA fragmentation, and the formation of membrane-enclosed structures termed "apoptotic bodies" that are extruded into the extracellular milieu (Wyllie *et al.*, 1980; Savill *et al.*, 1993). Recognition of these cellular corpses and their removal by phagocytosis are carefully controlled and occur without disturbance to tissue architecture or function and without initiating inflammation (Savill *et al.*, 1993).

Apoptosis can be induced by a variety of means such as DNA damage, growth factor deprivation, Fas/TNF/Reaper, loss of adhesion, oxidative stress or developmental cues (Rowan and Fisher, 1997). These triggers can then activate various positive and negative apoptotic modulators (*e.g. p53*, *c-Myc*, the *Bcl-2* family of proteins, cell cycle regulators, transcription factors) and the interplay between them defines the net cellular outcome (life or death). In the event a cell chooses to die, apoptotic machinery will be activated. Emerging evidence indicates that apoptosis utilises some of the same signal transduction mediators previously implicated in other physiological cellular responses, including intracellular Ca^{2+}, protein kinases and phosphatases, cAMP, lipids, pH and oxidative radicals (McConkey and Orrenius, 1996). A breakthrough in understanding apoptosis came in the recognition that, in all known instances of apoptosis, a family of cysteine proteases related to the IL-1β-converting enzyme (ICE) becomes activated and executes apoptosis through specific cleavage of substrates, now popularly know as death substrates (Martin and Green, 1995). One of the first recognised and common class of death substrates are endo-exonuclease precursors whose proteolytic cleavage causes enzymatic activation (Fraser 1994). Seeing apoptosis as a proteolytic process has revolutionised the way apoptosis is detected and studied, and apoptosis is now

commonly measured as proteolytic enzyme activity which facilitates the development of these cell-free apoptosis systems.

5.2. The *Bcl-2* family of proteins.

Bcl-2 is the founding member of an expanding family of proteins involved in the regulation of apoptosis. The human *bcl-2* gene was identified at the breakpoint site of the t(14;18) chromosomal translocation, which is associated with follicular lymphoma and results in the transcriptional activation and over-production of the 26 kD *Bcl-2* protein (Tsujimoto *et al.*, 1985). It proved to be a novel proto-oncogene: although *Bcl-2* alone does not stimulate cell proliferation or cause transformation, it co-operates with *c-Myc* (Vaux *et al.*, 1988; Fanidi *et al.*, 1992) and members of the *Ras* family (Reed *et al.*, 1990) to transform cells. Transgenic mice that overexpress *bcl-2* display increased tumour development (Korsmeyer 1995). In particular, when the *bcl-2* transgenic mice were mated to the *c-myc* transgenic mice, a rapidly emerging undifferentiated haemopoietic leukaemia occurred, demonstrating the potent synergy of this oncogene combination (Strasser *et al.*, 1990). Being an oncoprotein, *Bcl-2* proved to have novel unusual properties acting to promote survival rather than proliferation in cellular assays (Vaux *et al.* 1988; Hockenbery *et al.*, 1990). A rapid succession of experiments demonstrated that *Bcl-2* acts as an inhibitor of apoptosis in most of *in vivo* and *in vitro* systems with few exceptions (Reed 1994; Vaux *et al.*, 1992).

The *Bcl-2* protein has a fairly wide tissue distribution within adult tissues, including lymphoid, haemopoietic, epithelial and neural tissues (Hockenbery *et al.*, 1991). However, within these tissues, the *bcl-2* expression is limited to distinct cell types or topologic areas, and appears to be expressed in cells that are rapidly dividing and differentiating into mature components. *Bcl-2* is expressed more widely in foetal tissues, most notably in the central nervous system, where most populations of neurons express *bcl-2* at relatively high levels (Veis-Novack and Korsmeyer, 1994). Within the cells, the *Bcl-2* protein is located in nuclear and endoplasmic reticulum membranes and the outer membranes of mitochondria (Hockenbery, 1995). The *bcl-2*-deficient mice complete embryonic development and appear normal during the first week after birth (Veis-Novack *et al.*, 1993). Their spectrum of haemopoiesis is initially normal, so as development of most organs determined by histological analysis. Thus, the *Bcl-2* activity is not absolutely required for embryogenesis and development. However, abnormalities (including growth retardation, polycystic kidney disease, abnormal hair pigmentation, fulminant apoptosis of the thymus and spleen, and an almost complete loss of lymphocytes) develop over time and the mice die young. This illustrated that *Bcl-2* may have its most dramatic role in maintaining homeostasis in adult tissues.

Since the cloning and characterisation of *bcl-2*, a number of genes that share sequence homology with *bcl-2* have been isolated, which include *bcl-x*, *bax*, *mcl-1*, *A1*, *bag*, *bak* (Nunez and Clarke, 1994; Farrow and Brown, 1996). Members of this family and its interacting partners have been central to models that attempt to explain the mechanisms of apoptosis in mammalian cells. The homology among the *Bcl-2*-related proteins is concentrated in two regions, termed BH1 and BH2, which turn out to be important for homo- and hetero-dimerisation (Yin *et al.*, 1994). As it will be discussed below (see Section 5.4), homo- and hetero-dimerisation among the *Bcl-2* family proteins play a pivotal role in the regulation of their functional activities. In addition, proteins of the *Bcl-2* family contain a stretch of hydrophobic amino acids at their C-termini, which appear important for attachment to intracellular membranes (Nunez and Clarke, 1994). Indeed, *Bcl-2*-related proteins are found at similar locations in the cell as *Bcl-2* (Farrow and Brown, 1996). It is conceivable that, in each location, the cellular targets of *Bcl-2* family proteins are different, but are coupled to a common effector.

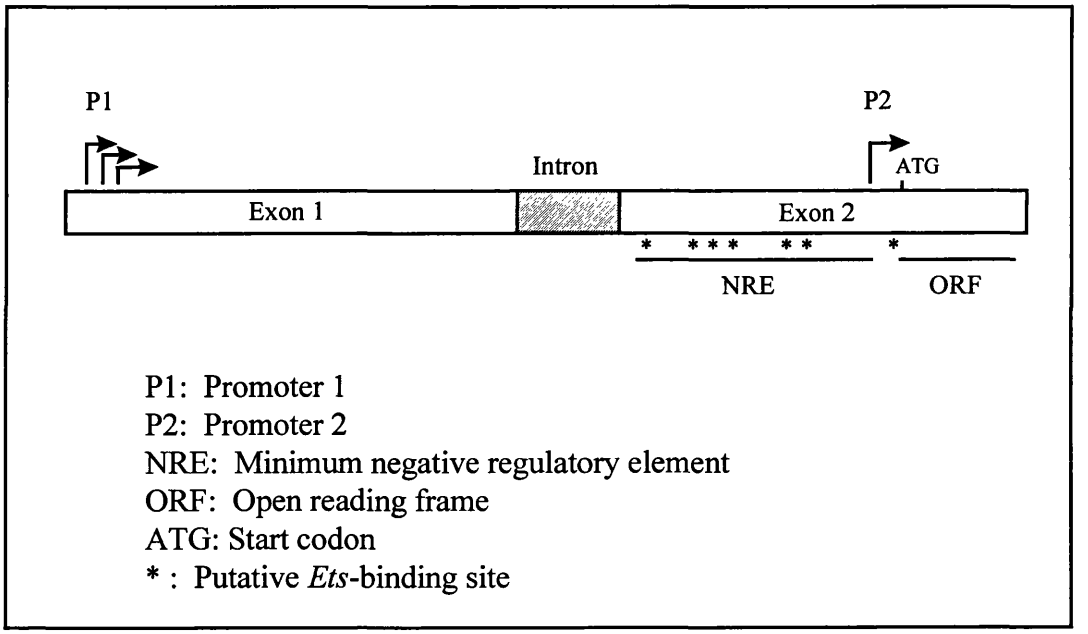
5.3. Transcriptional regulation of the *bcl-2* expression.

Although *bcl-2* is subject to both tissue-specific and developmental regulation, little is known about the molecular mechanisms controlling its expression. Characterisation of its genomic organisation revealed that *bcl-2* consists of three exons, with the second and third exons separated by extremely large, 225kb intron (Negrini *et al.*, 1987; Seto *et al.*, 1988). Two promoters are responsible for the initiation of *bcl-2* transcription (Figure 5.1). The predominant promoter, P1, is a GC-rich, TATA-less promoter that displays multiple start sites and includes several consensus binding sites for the *Sp1* transcription factor. This promoter structure is somewhat surprising for a developmentally regulated gene and is more commonly associated with constitutively expressed genes. The second promoter, P2, is located approximately 1.4kb downstream from the first, and includes both a CCAAT box and TATA element.

The unusual feature of the *bcl-2* gene is characterised by the very long (5' about 1.4kb and 3' about 5.1kb) untranslated regions (UTR) surrounding the relatively short coding region. More interestingly, the 5' and 3' UTRs are highly homologous between human and mice with 66% and 60% nucleotide sequence homology, respectively. It is conceivable that the UTRs might play a role in transcriptional regulation of the gene. Indeed, Young and Korsmeyer (1993) took advantage of the differential expression of *bcl-2* within the B-cell lineage and identified a novel negative regulatory element (NRE) in the human bcl-2 5' UTR that decreased expression from the *bcl-2* P1 promoter or heterologous promoters. Most of the negative regulatory activity of the 1.4kb NRE has been mapped to the 271bp region immediately upstream of the ATG start codon, and so this region is considered as the minimum NRE (see Figure 5.1). The minimum NRE

contains the *bcl-2* P2 promoter, an octamer mitif, and a cluster of putative *Ets*-binding sites (Figure 5.1). However, the NRE activity is similar in pre-B-cell and mature B-cell lines, that display different *bcl-2* expression at the transcriptional level. It was subsequently reported that *p53* can down-regulate *bcl-2* transcription via this element (Miyashita *et al.*, 1994). Interestingly, Frampton *et al.* (1996) identified several *Myb*-binding sites within the minimum NRE whereby *v-Myb* up-regulates *bcl-2* transcription. Therefore, although the NRE activity is an important determinant of the differential expression of *bcl-2*, it alone does not explain the developmental control of *bcl-2* transcription. At least one other mechanism emerging is the interplay between various positive and negative transcription factors that are able to bind to the NRE and control transcription of the gene.

Figure 5.1. Schematic representation of the *bcl-2* promoter.



5.4. Mechanisms and regulation of the *Bcl-2* functional activities.

Bcl-2 can inhibit apoptosis in a variety of cells and tissues across divergent phyla in response to a variety of stimuli. This indicates that *Bcl-2* must act at a final common pathway that operates in all forms of apoptosis and appears to be evolutionarily conserved. The mechanism whereby *Bcl-2* inhibits apoptosis is not clear but more and more direct evidence have arisen from recent discoveries. The first clue has come from studies of Hockenbery *et al.* (1993) and Kane *et al.* (1993), who postulated that *Bcl-2* inhibits cell death by acting as an antioxidant or inhibiting the generation of oxygen free radicals. Shimizu *et al.* (1996) also showed that induction of apoptosis by a Ca^{2+} ionophore or hypoxia results in increased levels and activity of active ICE-like cysteine proteases, the inhibition of which reduces the extent of cell death. Interestingly, over-

expression of *bcl-2* or *bcl-x*$_L$ inhibits apoptosis and the activation of ICE-like proteases, indicating that *Bcl-2* and *Bcl-x*$_L$ act upstream of these proteases. Marin *et al.* (1996) suggested that the anti-apoptotic effects of *Bcl-2* may, in part, be to prevent a sustained increase in nuclear and cytosolic Ca^{2+} following apoptosis induction.

Regulation of the *Bcl-2* functional activity can occur at either transcriptional/translational or post-translational level. It has been well established that a variety of growth factors can act as survival factors and prevent apoptosis in certain growth factor-dependent cells. In the case of IL-2, IL-3 and GM-CSF, it was reported that they do so by up-regulating *bcl-2* expression at the mRNA and protein levels (Duke and Cohen, 1986; Rinaudo *et al.*, 1995; Bradbury *et al.*, 1994). Kinases appear to be required for this activity since either herbimycin A, a protein tyrosine kinase inhibitor, or inhibitors of protein kinase C block the ability of IL-2 and IL-3 to up-regulate *bcl-2* expression and induce apoptosis (Rinaudo *et al.*, 1995; Otani *et al.*, 1993). In addition, there is evidence that growth factors induce the *bcl-2* expression through activation of the *Ras* signalling pathway (Kinoshita *et al.*, 1995). Stromal cells can also regulate the *bcl-2* expression in a stroma-dependent pro-B cell line. When removed from stromal cell cultures, the pro-B cells rapidly lose the *bcl-2* mRNA expression, which is coincident with immediate initiation of apoptosis (Gibson *et al.*, 1996). More direct evidence with regard to regulation of *bcl-2* expression comes from studies of *p53* and *v-Myb*. It has been reported that *p53* induces apoptosis by decreasing the *bcl-2* expression, and mice deficient in *p53* exhibit an increase in the *Bcl-2* protein in several tissues (Miyashita *et al.*, 1994b). Frampton *et al.* (1996) reported that *v-Myb* up-regulates *bcl-2* and suppresses apoptosis in myeloid cells. More importantly, both *p53* and *v-Myb* appear to act directly on the *bcl-2* promoter (Miyashita *et al.*, 1994a; Frampton *et al.*, 1996; also see Section 5.3).

In addition to modulate *bcl-2* expression, the *Bcl-2* activity can also be regulated, and this occurs either by phosphorylation or interaction with other proteins. Haldar *et al.* (1995) reported that *Bcl-2* can be phosphorylated on serine, and the phosphorylated protein can no longer protect cells from apoptosis. One way to induce *Bcl-2* phosphorylation is by microtubule depolymerisation (Haldar *et al.*, 1997) and, hence, it was proposed that *Bcl-2* may act as "guardian of microtubule integrity". *Bcl-2*-interacting proteins include the *Ras*-related protein, *R-Ras* p23, and the *Bcl-2*- related protein *Bax*. Identification of *R-Ras* as *Bcl-2*-interacting partner provides evidence of a putative component of a signal transduction pathway involved in the regulation of apoptosis (Fernandez-Sarabia and Bischoff, 1993), but direct evidence is lacking with regard to how this interaction may regulate the anti-apoptotic activity of *Bcl-2*. On the other hand, it has been reported that interaction of *Bcl-2* with *Bax* abolishes its anti-apoptotic activity (Oltvai *et al.*, 1993). When *Bcl-2* is in excess, *Bcl-2* homodimers

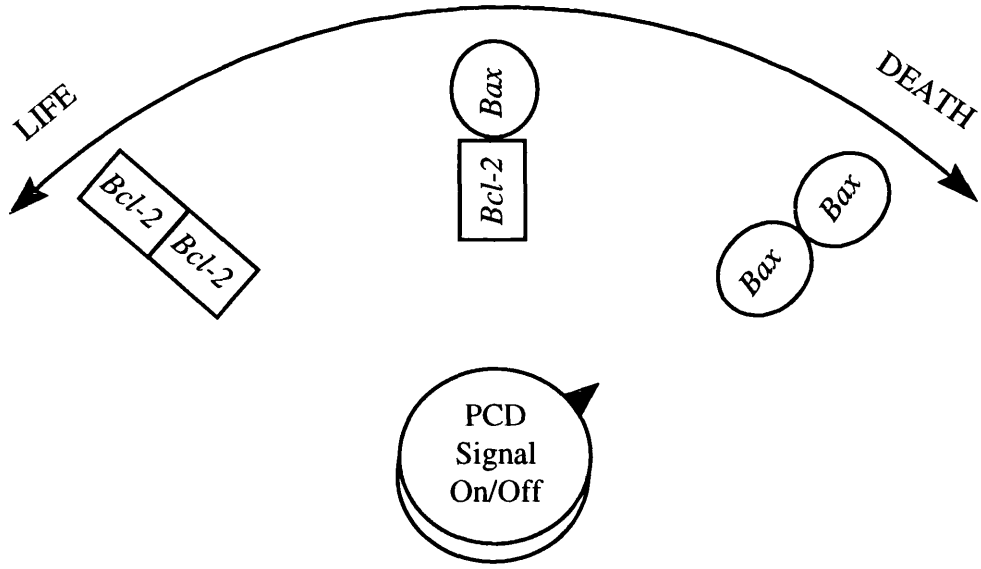
dominate and cells are protected. When *Bax* is in excess Bax homodimers dominate and cells are susceptible to apoptosis (Figure 5.2). Thus, the ratio of *Bcl-2*/*Bax* represents a cell-autonomous rheostat that pre-determines a cell's life or death response to an apoptotic stimulus. Of note, with the rapid increase of identification of *Bcl-2* family members and their protein partners, more and more proteins are being found that interact directly with either *Bcl-2* or *Bax* or both (Farrow and Brown, 1996), and so our understanding of the roles of *Bcl-2* family members is rapidly changing and the above picture is very simplified.

5.5. The roles of *Bcl-2* family proteins in regulating proliferation and differentiation.

Although *Bcl-2* family proteins were initially identified as regulators of apoptosis, recent evidence suggests that they may also play a role in co-ordinating cell proliferation and differentiation. Miyazaki *et al.* (1995) demonstrated that *Bcl-2* mediates IL-2 signalling in conjunction with either the *c-fos*/*c-jun* induction pathway involving *src* family protein tyrosine kinases or the *c-myc* induction pathway. Marvel *et al.* (1994) suggested a direct involvement of *Bcl-2* in cell cycle events, based on their demonstration that over-expression of *bcl-2* results in a delay in re-entry into cell cycle after growth factor withdrawal, implying that *Bcl-2* function may antagonise a critical event in G0/G1 transit after a period of quiescence.

The involvement of *Bcl-2* in regulating differentiation was first implied by its expression pattern within tissues. As mentioned above (see Section 5.2), the *bcl-2* expression in adult tissues appears to be restricted in cells that are rapidly dividing and differentiating into mature components. Such cells include stem cells of the crypts of the gut epithelia or the skin, and early haemopoietic progenitors. In some cases, the *bcl-2* expression declines in cells as they mature, or at stages when cells may be eliminated. For example, *bcl-2* is down-regulated during keratinocyte or myeloid differentiation (Hockenbery *et al.*, 1991), and also transiently during B- and T-cell differentiation at a stage when such cells are prone to undergo clonal elimination by apoptosis (Gratiot-Deans *et al.*, 1993; Merino *et al.*, 1994). Furthermore, down-regulation of the *bcl-2* expression in a epidermal keratinocyte cell line using an antisense approach results in a spontaneous epidermal differentiation (Marthinuss *et al.*, 1995). However, an up-regulation of *bcl-2* expression has also been documented in differentiating cells. Zhang *et al.* (1996) reported that the *bcl-2* expression increases in Paju cells, a human neural crest-derived tumour cell line, that are induced to undergo neural differentiation in response to phorbol ester. Over-expression of the *bcl-2* cDNA induces extensive neurite outgrowth, even in low serum concentrations, together with an increased expression of a neuron-specific enolase; whereas Paju cells expressing the antisense *bcl-2* cDNA

Figure 5.2. A pre-set rheostat model as to how the ratio of *Bcl-2*/*Bax* determines the life or death response of a cell following a programmed cell death (PCD) signal.



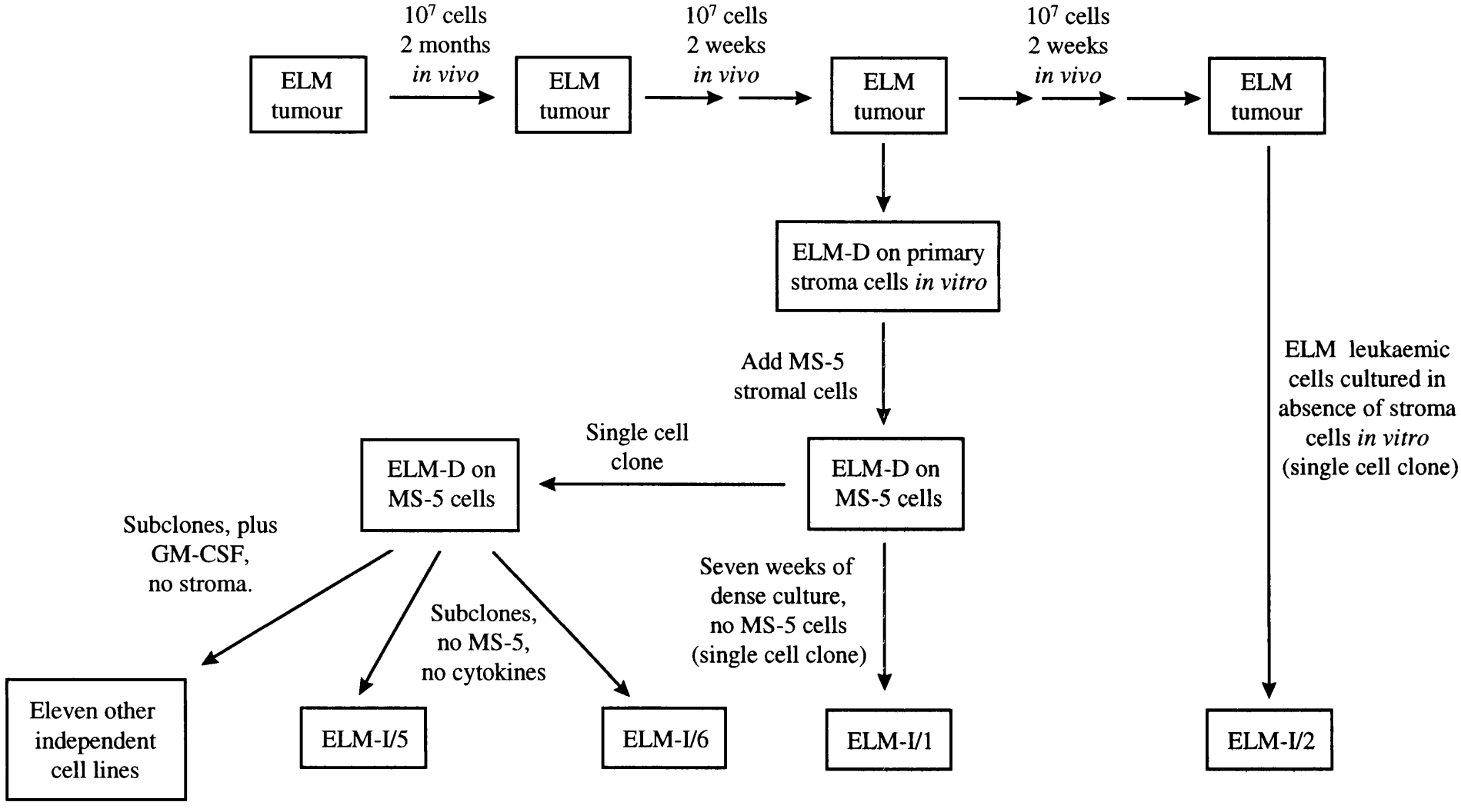
construct do not undergo spontaneous neural differentiation. Similarly, in a myeloid precursor cell line, expression of the *bcl-2* family member, *A1*, is stably increased during myeloid differentiation induced by G-CSF (Lin *et al.*, 1993). Finally, expression of another *bcl-2* family member, *mcl-1*, undergoes an early rapid increase (1-3h) followed by a later gradual fall in a human myeloid leukaemia cell line, ML-1, undergoing myeloid differentiation (Kozopas *et al.*, 1993). It appears, therefore, that quantitative changes in expression of *bcl-2* family members is required in various differentiation processes. The up- or down-regulation of *bcl-2* family members in different differentiation processes may reflect a difference in the mechanisms whereby differentiation is regulated.

Chapter 6: The ELM Erythroleukaemia System

6.1. Erythroleukaemia induced by X-ray irradiation.

A disease with the characteristics of an erythroblastic leukaemia, termed the ELM erythroleukaemia, was induced after a long delay in a female C3H mouse by X-ray irradiation of 300 Rads when the animal was 10 weeks old (Figure 6.1) (Itoh *et al.*, 1988a). Hepatomegaly, spenomegaly and anaemia were noticed after a ten month latency. Subsequent passage *in vivo* in the spleen generated erythroleukaemia within two weeks of injection, implying selection for more leukaemogenic cell clones during these early passages. Cytological analysis of peripheral blood from animals with a well-developed leukaemia showed that it consisted almost entirely of erythroid cells at different stages of differentiation. It is unusual for irradiation to induce erythroid malignancies in mice: usually cells from the lymphoid and other myeloid lineages are affected, and the genetic background of the irradiated host, to some extent, determines the nature of the haemotological malignancies formed (Janowski and Boniver, 1986; Mole, 1986). In some cases, leukaemia progression involves the production of recombinant endogenous retroviruses that are leukaemogenic in other non-irradiated host animals. Itoh *et al.* (1988a) performed a number of experiments that appeared to exclude any endogenous or exogenous retroviral involvement in the ELM tumour. First, both cell-free medium from short-term ELM spleen cell cultures and ultra-sonicated ELM cells failed to induced erythroleukaemia in 8-10 week-old C3H mice, demonstrating that no leukaemogenic retroviruses are being produced by the cells. Second, reverse transcriptase (RT) activity, which is present in retrovirus-producing cells, was not detected in the spleen and liver of mice with advanced ELM erythroleukaemia: the same assay had been used effectively to detect RT activity in Friend virus-infected cells (Ono *et al.*, 1984). These results distinguished ELM from Friend and Rauscher virus-induced erythroleukaemias and implied that the X-ray irradiation was the sole factor involved in initiating this malignancy, presumably by generating mutation in cellular genes. Autocrine growth factor production has been recorded in a number of haemopoietic malignancies (Sawada *et al.*, 1986; Meeker *et al.*, 1990). However, conditioned medium from short-term ELM leukaemic spleen cell cultures failed to increase erythroid burst formation from normal bone marrow cells, demonstrating that erythroid stimulatory factors, such as Epo, were probably not produced by the leukaemic cells as a result of these putative mutations (Itoh *et al.*, 1988a).

Figure 6.1. Derivation of the cell lines within the ELM system



6.2. Long-term growth *in vitro* supported by stromal cells.

Attempts to culture the spleen cells *in vitro* from the ELM tumour proved to be difficult, and even with IL-3, GM-CSF or Epo supplements, no cell lines could be established. This is quite distinct from the late stages of Friend erythroleukaemia, from which cell lines are readily generated even in the absence of growth factors. However, after an initial decrease in viability, ELM cells grew well on the stroma, initially on bone marrow-derived primary stromal cells, then later, on a bone marrow-derived stromal cell line, MS-5 (Itoh *et al.*, 1988b and Figure 6.1). Single cell-cloning of those leukaemic cells grown on stroma gave rise to the ELM-D cell line, with the "D" representing the stroma-dependence of the erythroleukaemia cells. The MS-5 cells are pre-adipocytes established after 900 Rads X-ray irradiation of a long term bone marrow culture (LTBMC) (Itoh *et al.*, 1988b; Suzuki *et al.*, 1992). They are capable of supporting the adhesion-dependent growth of haemopoietic stem cells and their progeny for greater than two months in LTBMC (Itoh *et al.*, 1989). Electron microscopic analysis showed that ELM-D cells adhere intimately to the stromal cells, with regions of extremely close contact (~10 Angstroms).

The stroma-dependence of ELM-D cells was rigorously tested by serial re-cloning experiments in the presence or absence of stromal cells. Clonal growth of ELM-D cells in the presence of stromal cell is very efficient but can be reduced by a factor of 10^4 when the feeder layer was omitted. However, during the first couple of weeks of cloning, many large colonies would arise that later disintegrate. The frequency of true stroma-independent variants is $<2 \times 10^{-5}$ (Prof. W. Ostertag, personal communication). These results confirmed that ELM-D cells show a genuine dependence on the stroma for long-term growth and survival, but are capable of proliferative cell expansion for a short term without stroma (2-3 weeks). Further evidence indicates that this stroma-dependence strictly requires direct cell-cell contact: (1) the long-term growth of ELM-D cells can not be supported by a cocktail of soluble growth factors including IL-3, GM-CSF and SCF, nor can conditioned medium from ELM-D/MS-5 co-cultures or MS-5 cells; (2) the long-term growth of ELM-D cells, when grown separated with the stromal MS-5 cells via an agar layer, is not maintained.

Dependence on specific growth factors for the survival and proliferation of leukaemic cells *in vitro* has been demonstrated in many studies, but the use of stromal cells to support growth has rarely been recorded. Bearing in mind the importance of the stromal cells in the maintenance of haemopoiesis (see Chapter 1 and Section 2.1), such stroma-dependent cell lines as ELM-D cells provide a unique and appropriate means of stromal cell investigation *in vitro* because: (1) morphological and kinetic analysis can easily be

performed on the cell populations; and (2) deletion and reconstitution experiments of putative functional stromal cell components can be carried out.

6.3. Stroma-independent derivatives.

As mentioned above (see Section 6.2), the ELM-D cell population contains a small proportion of cells that can grow without stromal cells and the frequency of occurrence of stroma-independent clones is approximately 2×10^{-5}. Fifteen stroma-independent ELM cell lines have been established by cloning or growth of ELM-D cells in the absence of stroma (Itoh *et al.*, 1988b and Figure 6.1), which are termed ELM-I cells with the "I" representing the stroma-independent growth of these cells. ELM-I/1 was derived from the non-adherent cell population from a dense culture of ELM-D cells grown on feeders for seven weeks. ELM-I/2 arose after culturing the ELM tumour cells (that had been passaged *in vivo*) directly *in vitro* in the absence of stromal cells. The remaining ELM-I cell lines were independently derived from the clonal ELM-D cell line, by selection for growth in suspension in the presence (eleven cell lines) or absence (I/5 and I/6) of GM-CSF. However, continued growth of those eleven cell lines proved not to be dependent on GM-CSF. In this thesis, ELM-I/1, I/2, I/5 and I/6 cells were routinely used.

6.4. Tumorigenicity and differentiation capacities.

The original ELM tumour can be passaged *in vivo*. Subsequently, a comparison of tumorigenicity of stroma-dependent and -independent ELM cells was performed by Itoh *et al.* (1988b) and Nibbs (unpublished data). This was carried out by injecting cells via the tail vein into syngenic C3H mice that had been immuno-suppresed by irradiation with 300 Rads of γ-rays. Mice were sacrificed 15-20 days post-injection and their spleens weighed as a measure of leukaemogenicity. It proved that all the ELM cells tested (ELM-D, ELM-I/1, I/2, I/5 and I/6) cause splenomegaly in mice and this is accompanied by liver enlargement. In addition, spleens from mice injected with ELM cells are also significantly paler than those from normal animals, indicating that severe anaemia might be induced in those mice. Most interestingly, ELM-I/1 cells consistently show the highest tumorigenicity, with the spleens up to eight times that of a normal spleen; whereas spleens from mice injected with other ELM cells are 2-3 times above the normal level. Moreover, mice injected with the ELM-I/1 cells also appear least healthy prior to sacrifice.

In parallel with these *in vivo* tumorigenicity assays, differentiation capacity of ELM cells was also tested in response to physiological growth factors, Epo and IL-3 (Nibbs *et al.*, 1993). The results showed that all of ELM cells apart from one (ELM-I/1) undergo

erythroid differentiation, indicated by an increase in the *α-globin* mRNA, in response to either Epo or IL-3. However, ELM-I/1 cells are insensitive to Epo or IL-3 induction. Later studies carried out by Dr. N. Leslie in our laboratory showed that ELM-I/1 cells can neither be induced to undergo erythroid differentiation by chemical inducers such as DMSO and HMBA. Taken together, ELM-I/1 cells seem to be blocked in differentiation and this may be associated with their high tumorigenicity *in vivo*.

6.5. Genetic characterisation.

Attempts were made to define the genetic changes responsible for the different phenotypes within the ELM system with regard to stroma-dependence, differentiation capacity and tumorigenicity (Nibbs *et al.*, 1993). At the *p53* locus, in both the primary tumour and all the ELM cell lines derived from it, one copy of the gene has been lost whereas the other contains an 18bp deletion, implicating its mutation as an early step in the development of the leukaemia. However, the mutation of *p53* found in these cells is not sufficient to confer stroma-independent growth or induce differerentiation arrest.

Although none of the ELM cells express the *c-ets-1*, *c-ets-2* or *spi-1/PU.1* mRNA, changes in gene expression of another two *ets* family members, *erg* and *fli-1*, were identified (Nibbs *et al.*, 1993). Only very low or undetectable levels of the *erg* mRNA are present in the primary leukaemic cells or ELM-D cells, whereas all the ELM-I variants have moderate levels of *erg* expression. Upon stroma-withdrawal, ELM-D cells show an elevated expression of the *erg* mRNA, yet, ELM-I cells do not down-regulate the *erg* mRNA when grown in contact with the stroma. This implies that up-regulation of *erg* expression seems to be important for stroma-independent growth and this seems to be made permanent in the ELM-I mutants.

The *fli-1* expression at the mRNA level is not detectable in any of the ELM cell lines apart from one (ELM-I/1), in which the *fli-1* expression is significantly up-regulated. Surprisingly, it was found that the *fli-1* gene region is rearranged not only in the ELM-I/1 varient espressing *fli-1*, but also in the primary tumour and all the ELM cells derived from it. The rearrangment is due to the insertion of an unexpected retrovirus inserted upstream of one *fli-1* allele, but this does not result in *fli-1* gene activation in any of the ELM cell lines except ELM-I/1. It appears, therefore, that insertion at the *fli-1* locus may be an important event in the evolution of this erythroleukaemia. The fact that the tumour cells are monoclonal with respect to this insertion implies that it was selected for during tumour development. There are precedents in other systems for such insertion being selected because of activation or inactivation of adjacent genes. In the ELM leukaemia, inactivation seems unlikely, since there is no evidence for *fli-1* expression in normal erythroid progenitors. Furthermore, only one allele appears to be affected by mutation in

the ELM tumour. There seem to be two plausible interpretations of the data involving gene activation by retroviral insertion. First, *fli-1* could have been activated in the original tumour but then became inactivated during subsequent passages *in vivo*, i.e., *fli-1* expression was required for initiation of the leukaemia but not for its subsequent maintenance. Alternatively, the retroviral insertion upstream of the *fli-1* gene could be activating another important gene nearby. A further question concerns the mechanism whereby *fli-1* becomes expressed uniquely in ELM-I/1 cells. No structural differences have been detected at the *fli-1* locus between ELM-I/1 and other ELM cell lines, although it cannot be excluded that small sequence changes may be affecting expression of the *fli-1* gene. Alternatively, ELM-I/1 may express certain transcription factors absent from other ELM cell lines that permit *fli-1* transcription.

6.6. Aims and objectives

As discussed above, the ELM system consists of a series of erythroleukaemic cell lines that are different in their stroma-dependence, differentiation potential in response to physiological growth factors, and tumorigenecity *in vivo*. Thus, the ELM system not only provides us a very useful cellular system that allows us to identify the cellular and molecular mechanisms of stroma-dependent growth, but also is an *in vitro* model of tumour progression and enables us to identify genetic changes involved in the occurrence of stroma-independent growth, differentiation arrest and increased tumorigenicity.

There were mainly three parallel aims of this project. The first aim was to characterise cellular and molecular events in ELM-D cells upon stroma-withdrawal in an attempt to understand the nature of stroma-dependent growth and the underlying molecular mechanisms. In particular, experiments were performed to determine whether apoptosis is the form of cell death that occurrs in ELM-D cells in the absence of stroma, and if genes that regulate apoptosis, such as *bcl-2*, may play a role in regulating stroma-dependent growth.

The second aim was to identify genetic changes that were associated with different phenotypes of ELM cells. The genes to be examined can be classified into three categories: (1) genes involved in the induction of erythroleukaemia (*erg* and *fli-1*); (2) genes involved in the control of cell survival, especially when in the absence of survival and proliferation signals that are normally present (*bcl-2* and *bcl-x*); and (3) genes that play a crucial role in the regulation of erythroid differentiation (*gata-1* and *scl*). If any particular gene appeared to be associated with a particular phenotype then functional work would be carried out to perturb gene expression by either over-expression or antisense approaches.

The third aim was to dissect the molecular mechanisms responsible for differentiation arrest and increased tumorigenicity associated with ELM-I/1 cells, especially in light of the fact that the *fli-1* mRNA is significantly up-regulated in this particular cell line.

PART II: METHODS AND MATERIALS

CHAPTER 7: Methods

7.1. Cell culture.

7.1.1. Culturing of mammalian cells in *vitro.*

All the ELM cell lines (Itoh *et al.*, 1988) and the MS-5 stromal feeder cells (Itoh *et al.*, 1989) were maintained in Minimal Essential Medium (α-MEM) in 5% (v/v) CO_2 at 37°C. α-MEM, containing deoxyribonucleosides and ribonulceosides, is supplemented with 16% (v/v) or 8% horse serum and 4mM glutamine. This medium will be referred to as the ELM growth medium hereafter. In long term cloning experiments, during which chances of contamination were significantly high, 100μg/ml of streptomycin and 37.5μg/ml penicillin were included.

MS-5 cells or ELM-D cells growing on live MS-5 cells were passaged as follows. Cells were washed with PBS (137mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH7.4) and then removed from the flask by treating with a 0.25% trypsin solution and incubating at 37°C for approximately 5min. After trypsinisation, 9 volumes of fresh ELM growth medium was added to dilute and thereby inhibit the trypsin. The resulting cell suspension was directly transferred into new flasks. All the stroma-independent cell lines of the ELM system (i.e. ELM-I cells) grow to some extent attached to the bottom of the tissue culture flasks. On passage, they were scraped off from the flasks using Disposable Cell Scrapers (Costar) and re-seeded at a density of 10^5 per ml in the fresh ELM growth medium.

The HL60, K562, KG-1 and Molt-4 cells were maintained in DMEM containing 10% (v/v) foetal calf serum and 4mM glutamine in 5% CO_2 (v/v) at 37°C. Since they are also suspension cells, and thus were passaged in a similar way as ELM-I cells.

The Q2BN cells were grown in DMEM containing 8% (v/v) foetal calf serum, 2% (v/v) heat-inactivated horse serum, 4mM glutamine, 0.375% (v/v) sodium bicarbonate, 1 mM sodium pyruvate and 10mM HEPES buffer in 5% CO_2 (v/v) at 37°C. This DMEM medium with supplement will be referred to as the Q2BN medium hereafter. The Q2BN cells were passaged as described for ELM-D/MS-5 cells.

7.1.2. Cell storage.

Stocks of cells were stored in liquid nitrogen. To freeze cell stocks down, cells were harvested as above, resuspended to a final concentration of 10^7 cells per ml in growth

medium containing 10% (v/v) DMSO and then dispensed into 1-2ml Nunc cryotubes and stored at -70°C for 24h in a well insulated container to give a rate of 1°C per min. Tubes were then transferred to liquid nitrogen for long-term storage.

To thaw frozen cell stocks, cells were placed in a 37°C water bath. As soon as they were thawed, they were diluted in 20ml of growth medium at 37°C and centrifuged for 5min at 1,200rpm in a MSE Centaur benchtop centrifuge. Cell pellets were then resuspended in fresh tissue culture medium and seeded into a tissue culture flask.

7.2. Growth curve analysis of ELM-D cells.

The ELM-D cells were grown to confluence on the stromal cells. They were then shaken off from the stroma, counted by a haemocytometer and diluted at a density of 10^5 per ml. 5ml of such cell suspension were aliquoted into the T25 flasks that had been seeded with 5 x 10^5 MS-5 feeder cells. At each time point, ELM-D cells were shaken off from the stroma and counted in a similar way. Each time course was performed in triplicates.

7.3. Cell viability assays

Cell viability assays were carried out using the trypan blue exclusion method (Sigma) which is based on the permeability changes of the cytoplasmic membrane that occur concomitantly with cell death, so that dead cells can uptake trypan blue and appear blue. To perform the assays, cell suspension was mixed with an equal volume of a trypan blue solution (Sigma) and the mixture was then incubated at room temperature for 5min. At the end of incubation, the number of blue cells and the total number of cells were counted using a haemocytometer.

7.4. Serial re-cloning of ELM-D cells.

In the serial re-cloning experiments, ELM-D cells were cloned on MS-5 cells irradiated with 17,000Rads of γ-rays from an Alcyon II Teletherapy Unit containing a ^{60}Co source. It has been demonstrated that the lethally irradiated stromal cells almost cease proliferation but can support the growth of ELM-D cells for up to 2-3 weeks before they disintegrate. The MS-5 cells were seeded in 96-well plates (Nunc) at a density of 2-3x10^3 cells per well. After irradiation, the medium was replaced with 100μl of fresh medium. To clone ELM-D cells, they were grown on the irradiated MS-5 cells to sub-confluence when they were shaken off from the stroma, counted using a haemocytometer and then re-seeded into 96-well plates containing irradiated stroma in such a way that there were 1, 3, 10 and 30 cells in 200μl of the ELM medium per well. At about day 10 after cloning, 100ul of fresh medium were added into each well with

care. After 2-3 weeks, the number of clones that grown out were counted and at least 8-10 clones picked and subsequently re-cloned as above. The cloning effiency was calculated as the number of clones grown out divided by the total number of cells seeded initially.

7.5. Staining ELM-D cells with Giemsa.

Approximately 1-2x10^5 cells in suspension were centrifuged onto microscope slides in a Cryo-Tek cytospin centrifuge at 500rpm for 5min, washed once with PBS (see Section 7.1.1) and fixed in ice-cold 70% ethanol for 1min. The fixed cells were then stained with a Giemsa solution (0.4% (w/v) in buffered methanol solution, pH6.8, Sigma) for 5min, washed 2-3 times with dH_2O, dried and finally mounted under coverslips with Depex.

7.6. Differentiation induction *in vitro*.

Cells were grown to sub-confluence, harvested and then re-seeded in the ELM growth medium at a density of 10^5 cells per ml plus either no additional growth factors or 1U/ml of recombinant human EPO (Boehringer Mannheim). Culture were incubated for 3h, 6h or 1-5 days and the RNA extracted (see Section 7.9.3).

7.7. Transient and stable transfection of Q2BN and ELM cells.

7.7.1. Transient transfection of Q2BN cells by the calcium phosphate precipitate method.

Calcium phosphate-mediated transfection of the adherent Q2BN cells was carried out as described in Sambrook *et al.* (1989). 24h before transfection, exponentially growing Q2BN cells were harvested by trypsinisation and re-plated at a density of 2x10^5 cells per 5ml per 60mm dish (Nunc). They were incubated for 20-24h in 5% (v/v) CO_2 at 37°C in a humidified incubator. Calcium phosphate-DNA precipitation was carried out as follows (the amounts given are sufficient for triplicate transfections): 770µl of DNA solution in 0.1xTE (1xTE: 10mM Tris.HCl , 1mM EDTA (pH8.0)) was added into 109µl of 2.46M $CaCl_2$ and mixed well, then 875µl of 2xHBS (2xHBS: 280mM NaCl, 10mM KCl, 1.5mM Na_2PO_4, 12mM glucose and 50mM HEPES (pH7.9)) was added with mixing by pipetting up and down rapidly 6 times. The mixture was left at room temperature undisturbed for exactly 20min, then 0.5ml of the calcium phosphate-DNA precipitate solution was transferred into each 60mm dish containing Q2BN cells in 5ml medium. After 24h incubation at 37°C, the medium was removed and 5ml of fresh medium added at 37°C. Assays for transient expression were carried out 24h later.

7.7.2. Transfection of ELM cells by electroporation.

24h before transfection, ELM cells were seeded at a density of $4x10^5$ cells per ml. For electroporation, cells were harvested, spun down and resuspended at a density of $2.5x10^5$ cells per ml in ELM growth medium. 200µl of such cell suspensions containing $5x10^6$ cells were used for each electroporation. 40µl of 0.5µg/µl DNA solution in 1xTE (see Sction 7.7.1) were gently mixed with the cell suspension in a electroporator cuvette and then equilibrated to room temperature for 10min. For generation of stably transfected ELM cells, electroporation was carried out at 960µF and 260V using a BioRad Gene Pulser with a capacitance extender. For transient expression assays, a much improved transfection efficiency was desired and this was achieved, to a limited extent, by using a Flowgen EasyjecT Plus electroporator at 1050µF and 280V. After electroporation, cells were immediately transferred to 6-well plates (Nunc) in 10ml of medium at 37°C and then incubated for 48h when transient expression assays (see Section 7.7.4) or selection of stably transfected cells (see below Section 7.7.3) were be carried out.

7.7.3. Selection of stably transfected ELM cells.

48h after electroporation, ELM cells were cloned in 96-well plates in the ELM growth medium containing 100µg/ml of streptomycin, 37.5µg/ml penicillin and 80mg/ml G-418 sulphate (Sigma) to select for cells expressing the transgene. Cells were plated by end-point dilution so that there were 300, 1000, 3000 cells in 200µl of medium per well. Such large number of cells were required because the transfection efficiency was very low and furthermore electroporation tends to cause large extent of cell death. Ten days later, 100µl of the growth medium containing appropriate antibiotics and G418 were added into each well with great care so as not to disturb the clones that might have already grown up. Another 5-10 days later, clones were counted and selected for expansion and further analysis.

7.7.4. Luciferase assays.

Luciferase assays were performed at 20°C throughout using a Luciferase Assay System purchased from Promega. Growth medium was removed from the cells which were then washed twice in PBS (see Section 7.1.1). The minimal volume of cell lysis reagent was added to the cells (e.g. 250µl for a 60mm culture dish in the case of adherent Q2BN cells or 100µl for each transfection in the case of ELM suspension cells) and the mix was incubated at 20°C for 10-15min. In the case of Q2BN cells, cells were scraped off from the culture dish using Disposable Cell Scrapers (Costar) and the cell lysates

transferred to microfuge tubes. Samples were spun at 14,000rpm in an Eppendorf microfuge for 2-3min and the supernatants then transferred to fresh microtubes. The luciferase activity of 20µl of cell lysates was measured by a luminometer.

7.7.5. β-galactosidase assays.

30-60µl of the cell lysates prepared as described above (see Section 7.7.4) were added into Solution I (40mM NaH_2PO_4, 60mM Na_2HPO_4, 10mM KCl, 1mM $MgCl_2$ and 50mM β-mercaptoethanol) followed by 0.1ml of Solution II (40mM NaH_2PO_4, 60mM Na_2HPO_4 and 2mg/ml ONPG) and the mixture was incubated at 37°C for 30-90min depending on transfection efficiency. The reaction was stopped by the addition of 0.25ml of 1M Na_2CO_3 and the absorbance at 420nm determined by a DU® 650 spectrophoto-meter (Beckman) was a measure of β-galactosidase activity.

7.8. Tumorigenicity assays *in vivo*.

To test the tumorigenicity of the ELM cell lines, cells were grown to sub-confluence and harvested. The viability of the cells was measured by the trypan exclusion method (see Section 7.3) and then 1-1.5x10^7 cells in 0.2ml of PBS were injected via the tail vein into recipient C3H mice that had been immuno-suppressed by irradiation with 300Rads of γ-rays from rays from an Alcyon II Teletherapy Unit containing a ^{60}Co source. After a latency period of about 2-5 weeks depending of the cell types, some animals became sick due to splenomegaly, when all the mice were sacrified. The spleens were removed after disecting any visible connective tissue. A group of 9 control mice of similar age, which had been injected with 0.2ml PBS (see Section 7.1.1), were also included.

7.9. Preparation of oligonucleotides, mammalian genomic DNA and total RNA.

7.9.1. Purification and precipitation of nucleic acid samples.

To remove protein contaminants, a combination of phenol-only (ΦOH), phenol-chloroform (ΦOH/$CHCl_3$) (50:50 (v/v) mix) and chloroform ($CHCl_3$) extractions were used. Phenol was prepared according to Sambrook *et al.* (1989). $CHCl_3$ was saturated with dH_2O. To extract proteins, an equal volume of these solutions was added to nucleic acid samples, vortexed and the layers separated by centrifugation either for 15min at 3,000rpm at 20°C in a Sorvall® Centrifuge RC3C (DuPont) using the swing-out rotor H6000A/HBB6 (large samples) or for 10min at 14,000rpm in an Eppendorf microfuge. Nucleic acids remained in the upper aqueous layer.

Nucleic acids were precipitated by the addition of 1/10th volume of 3M NaAc (pH5.2) and then 3 volumes of ethanol. This was mixed by vortexing and then precipitation carried out at -20°C for 0.5-1h. The nucleic acid was pelleted by centrifugation at 4°C for 25min at 3,000rpm in a Sorvall® Centrifuge RC3C using the swing-out rotor H6000A/HBB6 or for 15min at 14,000rpm in an Eppendorf microfuge. The pellets were washed once with ice-cold 75% ethanol, centrifuged again as above, air dried, and then re-suspended in the appropriate buffer.

7.9.2. Preparation of oligonucleotides.

Oligonucleotides were synthesised on an Applied Biosystems model 381A DNA synthesiser according to manufacturers' instructions. 5' trityp groups were removed by the machine and the DNA immobilised on a column. The DNA was eluted in 29% (v/v) ammonia by passing the solution through the column once every 5min for 1h. This solution was sealed in a glass vial and incubated at 55°C overnight to deprotect the oligonucleotides. Oligonucleotides were precipitated by adding 1ml of butan-1-ol to 100-150µl of the ammonia stock, the mixture incubated at 20°C for 10-15min and the oligonucleotides pelleted for 5-10min at 14,000rpm in an Eppendorf microfuge. The supernatant was discarded and the butanol was removed by a speedivac. The dried pellets were resuspended in 100µl of 1xTE buffer (see Section 7.7.1). Oligonucleotides were quantitated as described in Section 7.9.4.

7.9.3. Preparation of genomic DNA.

Cells were resuspended in 1xTE (see Section 7.1.1) to a final concentration of $5x10^7$ cells per ml and 10 volumes of extraction buffer (10mM Tris.HCl (pH8.0), 0.1mM EDTA (pH8.0), 0.5% (w/v) SDS and 20µg/ml DNase-free RNase) added. After a 60min incubation at 37°C, samples were mixed gently on a Stuart TR-2 tube rotator and proteinase K added to a final concentration of 0.1mg/ml. This was gently mixed and incubated at 50°C for 3h, swirling periodically. After cooling to room temperature, the protein fragments were removed by two phenol extractions. DNA was then ethanol precipitated (see Section 7.14.1) and collected by spooling it onto a clean Pasteur pipette moulded into a hook-shape over a Bunsen. The DNA pellets were allowed to air dry for 15min and then slowly resuspended at 4°C for 3 days in a volume of 1xTE (see Section 7.1.1) such that 1ml was used for every $2x10^7$ cells in the original sample. This solution was then dialysed in 1xTE (see Section 7.1.1) for 18h at 4°C to remove any remaining salt and ethanol, and was stored at 4°C. The DNA concentration was determined spectrophotometrically (see section 7.9.4).

7.9.4. Preparation of total RNA.

Total cellular RNA was prepared using the TRIzol method (Gibco), following the manufacturer's instructions. Cells were grown to sub-confluence and harvested followed by centrifugation at 1,200rpm for 5min in a MSE Centaur benchtop centrifuge. After removed of growth medium, the cells were lysed by dispersing the cell pellets in TRIzol Reagent at a density of 5-10x10^6 cells per ml followed by incubation at 20°C for 5min. Then 0.2ml of chloroform per 1ml of TRIzol was added and samples vortexed vigorously for 15sec and the incubation continued at room temperature for 2-3min. Samples were then centrifuged at 14,000rpm in an Eppendorf microfuge at 4°C for 15min and the upper colourless aqueous phase containing RNA was transferrred to a fresh tube. The RNA was precipitated by incubating with 0.5ml of isopropanol per ml of TRIzol Reagent at RT for 10min. Samples were then centrifuged as above for 10min and RNA pellets washed with ice-cold 75% ethanol using at least 1ml of 75% (v/v) ethanol per ml of TRIzol Reagent. RNA was spun down as above, the pellets air dried for 5min and dissolved in a buffer containing 10mM EDTA (pH8.0), 1% (w/v) SDS and 1μg/μl proteinase K. To facillitate dissolving, RNA samples were incubated at 55-60°C for 5-10min.

To avoid degradation by contaminating RNases, a number of precautionary steps were taken. First, plastic rather than glass ware was preferred for handling and storage of solutions. All tubes were autoclaved before use. Second, all solutions including ddH_2O were pre-treated with 0.1% (v/v) DEPC, an irreversible inhibitor of RNases, and autoclaved. Treatment with DEPC was carried out in a fume hood for 3h at 37°C or overnight at room temperature. Third, the buffer used to dissolve RNA contains SDS and proteinase K and this serves to inhibit any residual RNases or those that carried over during subsequent handling of the samples. Finally, all solutions were pre-cooled in ice and all manipulations and centrifugations were carried out at 4°C. The concentration was determined sepctrometrically (see Section 7.9.4.)

7.9.5. Quantitation of DNA and RNA.

Nucleic acid concentrations were determined spectrophotometrically. 3μl of the sample was added to 300μl of dH_2O and absorbance (A) readings taken at 260nm and 280nm in a quartz cuvette using a DU® 650 spectrophotometer (Beckman). dH_2O was used as the blank for quantitating DNA samples. The blank for RNA samples was prepared by adding 3μl of RNA dissolving buffer into 300μl dH_2O. An A_{260} value of 1 was taken to be equivalent to 50μg/ml of plasmid or genomic DNA, 40μg/ml of RNA and 20μg/ml of oligonucleotide. The A_{260}/A_{280} ratio was used as a measure of purity: samples giving a

ratio less than 1.75 were further purified by ΦOH/$CHCl_3$ extraction (see Section 7.12.1), ethanol precipitation and the A_{260}/A_{280} ratio re-assessed.

7.10. PCR from genomic and plasmid DNA.

The concentrations of DNA template used in PCR reactions were 40ng/μl for genomic DNA and 2ng/μl for plasmid DNA in 1x TE (see Section 7.7.1). 5μl were used for amplification. The primers used are listed in Table 7.1.

Table 7.1. Oligonucleotide primers for PCR from genomic DNA.

Name	Sequence	Position[α]
gata-1 F	CTCAGAACCCTTGCCCCAGTTTGTGGATTC	101
gata-1 R	AGTGGGGCGATCACGCTGGTGCTGCTGGTG	1271
bcl-2 F1	ACAGTAAGTTCTTTGCACAGGAATTTTGTT	1303
bcl-2 R1	CCTTCCCCGAAAAGAAGCTGCAGGTACAAC	1791
bcl-2 F2	TAACTTCCTAGGTCGTCCCGCCTCTTCACC	1491
bcl-2 R2	TCTCCCGGTTATCATACCCTGTTCTCCCGG	1831

[α]. The positions of the primers are based on the *gata-1* mRNA and *bcl-2* genomic sequences published by Tsai *et al.* (1989) and Negrini *et al.* (1987), respectively.

7.10.1. PCR using *Taq* DNA polymerase.

Amplification of partial gata-1 fragment from genomic DNA was carried out by polymerase chain reactions (PCR) using *Taq* DNA polymerase. The PCR reaction mix was set up as follows:

ddH_2O	29μl
10x PCR Reaction Buffer (Gibco)	5μl
25mM $MgCl_2$	3μl (final 1.5mM)
2mM dNTPs (Pharmacia)	2.5μl
DNA template	5μl
forward primer (140ng/μl)	2.5μl
reverse template (140ng/μl)	2.5μl
Taq DNA polymerase (10U/μl,Gibco)	0.5μl

The mixture was mixed thoroughly and 50μl mineral oil added to prevent evaporation. The reactions were carried out in a Perkin-Elmer Thermal Cycler 480 with the following program:

No. of programs	Temperature	Time	Cycles
1	95°C	2min	1
2	95°C 55°C 72°C	1min 1.5min 2min	25-30
3	72°C	10min	1
4	4°C	hold	-

The products were electrophoresed on an agarose gel (see Section 7.11.2) and the appropriate sized fragments were purified using the FMC SpinBind® DNA Recovery System (see Section 7.11.3). PCR products generated using Taq DNA polymerase were cloned by the TA Cloning® System (see Section 7.12.7).

7.10.2. PCR using *Pfu* DNA polymerase.

Amplification of the partial *bcl-2* promoter was carried out by polymerase chain reactions (PCR) using *Pfu* DNA polymerase. The PCR reaction mix was set up as follows:

ddH_2O	32.5µl
10x cloned Pfu buffer (Stratagene)	5µl
2mM dNTPs (Pharmacia)	2.5µl
DNA template	5µl
forward primer (140ng/µl)	2.5µl
reverse template (140ng/µl)	2.5µl
Pfu DNA polymerase (2.5U/µl, Stratagene)	1µl

The rest of the procedure was similar to that using *Taq* DNA polymerase apart from that the extension temperature was 42°C instead of 55°C (see above Section 7.10.1). PCR products generated using *Pfu* DNA polymerase were cloned by the pCR-Scrip™ .Amp.SK(+) Cloning kit (Stratagene, see Section 7.12.8).

7.11. Restriction digest and agarose gel electrophoresis of plasmid DNA.

7.11.1. Restriction digest.

Small quantities of plasmid DNA (<2µg) were digested in the appropriate buffer in a total volume of 20µl using 1-10 units of enzymes per ug of DNA, depending on the

enzyme used and the number of sites present. Larger, preparative digests were carried out proportionately larger volumes. For double digests, suppliers' information was consulted and the most appropriate buffer used. Reaction mixes were incubated for 2-3h at 37°C and then terminated by the addition of 1/6th volume of 6x DNA gel-loading buffer (6x buffer: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol).

7.11.2. Agarose gel electrophoresis.

DNA fragments were resolved on non-denaturing agarose gels containing ethidium bromide for subsequent visualisation under UV luminator. In general, 1% (w/v) agarose gels were used, but smaller fragments (100-400) were separated on 2-4% gels. Gel mixes containing the appropriate amount of agarose in 1xTAE (50xTAE: 2M Tris, 50mM EDTA and 57.1ml/l glacial acetic acid) were heated in a microwave oven to dissolve the agarose, and then cooled to 60°C when ethicium bromide was added to 5μg/ml and the mix was poured into appropriate gel former. Gels were allowed to set at room temperature and installed into the electrophoresis tank in 1xTAE buffer. Samples containing the DNA gel-loading buffer (see Section 7.11.1) were loaded and electrophoresed at around 100V. Molecular weight standards used include the 1kb ladder and Hae III-digested bacteriophage Φx174 fragments. A photograph was taken of the gel with a polaroid camera, using a TF-35M UV luminator (Vilber Laurmat).

7.11.3. Purification of DNA fragments from agarose gels.

DNA fragments separated from agarose gel were purified using the SpinBind® DNA Recovery System (FMC) following manufacturer's instruction. This system is able to recover DNA fragments from agarose gel ranging in size from 20bp to 5,000bp with an effective DNA capacity from 20ng to 30μg. Briefly, the volume of gel slices containing the DNA fragments of interest were estimated as its weight and 3 volumes of Binding Solution (7.2M NaI, 20mM DTT and 0.1M sodium phosphate (pH6.0)) added. The samples were incubated at 20°C for 10-15min or until the gel slices were completely dissolved and then were loaded into the SpinBind unit attached to microfuge tubes and incubated for 1min at RT to allow the solution to wet the membrane uniformly. The units were the centrifuged at 14,000rpm for 15sec in an Eppendorf microfuge. The filtrates in the microfuge tube were were re-applied to the Spin-Bind units and re-centrifuged as above. Finally, the binding process was repeated once more to ensure maximum binding of the samples. After the binding procedure, the SpinBind units were washed using 400μl of Sodium Iodide Wash Buffer (4.8M NaI, 20mM DTT and 0.066M sodium phosphate (pH6.0)) and spinning as above. The wash solution was discarded. The units were further washed three times using 400μl of ice cold High Ethanol Wash Buffer (50mM Tris.HCl, 1mM EDTA (pH8.0) and 80% (v/v) ethanol).

At the end of the washing, the empty units were spun at the maximum speed for 45sec to remove any remaining ethanol wash buffer and then air dried for 2min. The SpinBind units were transferred to fresh microfuge tubes and 50µl of 65°C DNA elution buffer (either 1xTE or dH20) applied, and spun as above for 15sec to recover the DNA samples.

7.12. Recombinant DNA techniques.

7.12.1. Host cells.

For propagating plasmids, DH5α *E.coli* cells were grown at 37°C either on inverted 1.5% (w/v) agar L-broth plates (L-broth: 10mg/ml bacto-tryptone, 5mg/ml bacto-yeast extracts, 171mM NaCl, pH7) or in Terrific broth (12mg/ml bacto-tryptone, 24mg/ml bacto-yeast extracts, 0.4% glycerol, 17mM KH_2PO_4 and 72mM K_2HPO_4) liquid medium supplemented with Amphicillin at a final concentration of 50µg/ml. Liquid cultures were grown in a New Brunswick G25 incubator shaker with a speed of 250rpm.

7.12.2. Preparation of competent cells.

To prepare competent DH5α cells, 2ml of an "overnight culture" was used to inoculate 200ml of L-broth (plus the necessary antibiotics), and then incubated at 37°C until the absorbance at 650nm was 0.5 units above an L-broth blank. These logarithmically growing cells were harvested by centrifugation at 2,000rpm for 10min at 4°C in an IEC Centra-8R centrifuge. The pelleted cells were resuspended in 80ml of RFI solution (100mM RbCl, 50mM $MnCl_2$, 30mM KAc, 10mM $CaCl_2$, 15% (v/v) glycerol, pH5.8), incubated on ice for 2h, pelleted as above and then resuspended in 16ml of RFII solution (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$ and 15% (v/v) glycerol). After incubating on ice for 15min, 0.8ml aliquots were transferred into 2ml Nunc cryotubes, frozen in a dry ice/ethanol bath and finally transferred to -70°C for storage.

7.12.3. Removal of protruding 3' termini from DNA fragments that have been digested with restriction enzymes.

In a 20µl reaction, 0.2-5µg of DNA were digested with the appropriate restriction enzymes (see Section 7.11.1). At the end of the digestion, 1µl of 2mM dNTPs was added followed by 1-2U of bacteriophage T4 DNA polymerase per µg of DNA, and the reaction mix incubated at 12°C for 12min, after which all the enzymes present in the reaction were inactivated by either heating to 75°C for 10min or the addition of equal volume of 1xTE (pH7.6, also see Section 7.7.1) followed by phenol:chloroform extraction and DNA precipitation (see Section 7.9.1).

7.12.4. Ligation of DNA fragments into plasmids.

Apart from when using the pCR-Script™.Amp.SK(+) cloning kit (see Section 7.12.8), ligation of DNA fragments into plasmids was carried out as follows. DNA fragments of interest obtained by either PCR (see Section 7.10) or restriction enzyme digest were purified as described in Section 7.11.3 and ligated to the desired linearised plasmid DNA as follows:

5x Reaction Buffer (Gibco, containing PEG)	2µl
DNA fragment	100ng
linearised plasmid DNA	50-100ng
T4 DNA ligase (Gibco, 1U/µl)	1µl
ddH_2O	make up to 10µl

The mixture was incubated at 12°C overnight, 40µl of either 1xTE (see Section 7.7.1) or ddH_2O added to dilute out PEG (which can interfere with transformation) and 5µl used for transformation (see below Section 7.12.5)

7.12.5. Transformation of bacteria.

Apart from when using the pCR-Script™.Amp.SK(+) cloning kit (see Section 7.12.8), transformation was carried out as follows. To propagate circular plasmid DNA in *E.coli* (see Section 7.12.1), 1-5µl plasmid DNA (1-10ng) were added into 50-100µl of competent bacteria DH5α cells, mixed by gentle swirl, and incubated on ice for 20min followed by heat shock at 42°C for exactly 45sec and then incubated again on ice for another 2min. Then L-broth (see Section 7.12.1) was added to make up the volume to 0.5-1ml and the content was incubated at 37°C for 1h in a incubator New Brunswick G25 incubator shaker with a speed of 250rpm. 10-100µl of baterial culture were then plated using a sterile glass spreader onto 1.5% (w/v) agar L-broth plates (see Section 7.12.1) containing 50µg/ml ampicillin. When blue/white selection was desired, 25µl of X-gal (40mg/ml stock in dimethylformamide) was also spread on top of agar plates. The plates were then incubated at 37°C overnight. In the case of blue/white selection, white colonies were chosen for further analysis.

7.12.6. Bacterial glycerol stocks.

The DH5α cells transformed with useful plasmids were stored as glycerol stocks for future retrieval. Stationary cultures in liquid medium were mixed with an equal volume

of a 30% (v/v) glycerol/L-broth solution (see Section 7.12.1), cooled on ice and then frozen at -70°C.

7.12.7. Cloning PCR products using TA-cloning kits.

This takes advantage of the non-template dependent activity of *Taq* DNA polymerase that adds a single deoxyadenosine to the 3' ends of duplex molecules. These 3' A-overhangs are used to insert the PCR product into a vector which contains 3' T-overhangs at its insertion site. Ligation and transformation were carried out as described above (see Sections 7.12.4 and 7.12.5).

7.12.8. Cloning PCR products using pCR-Script™.Amp.SK(+) cloning kit.

As *Pfu*-generated blunt-ended PCR fragments did not contain sites for restriction enzyme Srf, they were cloned using a pCR-Script™.Amp.SK(+) cloning kit (Stratagene). Ligation mix was prepared as the following order:

pCR-Scrip.Amp.SK(+) cloning vector (10ng/μl)	1μl
pCR-Scrip 10x reaction buffer	1μl
10mM rATP	0.5μl
PCR product	2-4μl
Srf restriction enzyme (5U/μl)	1μl
T4 DNA ligase	1μl
ddH_2O	make up to 10μl

The ligation mix was mixed gently and incubated at 20°C for 1h, then heat-inactivated at 65°C for 10min, and then stored on ice and ready for transformation into *E.coli* supercompetent cells supplied with the kit.

Duing transformation, 40μl of the supercompetent *E.coli* cells were pipetted into pre-chilled 15-ml Falcon 2059 polypropylene tube and 0.7μl of the β-mercaptoethanol added to yield a final concentration of 25mM. The mixture was swirled gently and placed on ice for 10min, swirling gently every 2min. 2μl of the DNA sample was then added into the bacteria, swirled gently and incubated on ice for 30min. The transformation mixture was heat-pulsed at 42°C for 45sec and then incubated on ice for another 2min. Then 0.45ml of pre-heated (42°C) SOC medium (20mg/ml bacto-tryptone, 5mg/ml bacto-yeast extract, 8.6mM NaCl, 2.5mM KCl, 10mM MgCl2, 20mM glucose, pH7) was added and samples were incubated at 37°C for 1h with shaking. Finally, 50μl, 100μl, 150μl and 190μl of the transformation mixture, together with 25μl of X-gal (see Section 7.12.5) and 10μl of ITPG (200mg/ml stock in ddH_2O) were plated

out on agar plates containing ampicillin. The plates were incubated at 37°C overnight and white colonies picked. For the control insert, X-gal and ITPG were not necessary and the antibiotic used was 30μg/ml of chloramphenicol instead of ampicillin.

7.13. Isolation of plasmid DNA.

7.13.1. Mini-preparations of plasmid DNA.

One step 'miniprep' method for the isolation of plasmid DNA was carred out according to Chowdhury (1991). 1.5ml of overnight bacterial culture in Terrific broth (see Section 7.12.1) was harvested in a microfuge tube and spun at 14,000rpm in an Eppendorf microfuge for 30sec. 1ml of supernatant was discarded, 0.5ml of PCI (phenol:chloroform:isoamylalcohol = 25:24:1) added and the mix vortexed and then transferred to a Eppendorf mixer for 5-10min. Samples were spun at 20°C for 5min and the upper aqueous layer transferred to fresh microfuge tubes containing an equal volume of isopropanol. After mixing thoroughly by vortexing, plasmid DNA and RNA was pelleted by centrifugation as above for 10min. The pellets were washed twice with ice-cold 75% (v/v) ethanol, air dried and re-suspended in 20μl of 1xTE (see Section 7.7.1) containing 20μg/ml RNaseA.

7.13.2. Large scale preparation of plasmid DNA.

30ml of overnight bacterial culture of *E.coli* in Terrific broth (see Section 7.12.1) were harvested in a 50ml-Falcon tube and spun at 4,000rpm for 10min at 4°C using a Sorvall Centrifuge RC3C using a H6000A/HBB6 swing-out rotor. The supernatant was discarded and the bacterial pellets re-suspended in 5ml Resuspension Buffer (50mM Tris.HCl (pH8.0), 10mM EDTA (pH8.0), and 100μg/ml RNase A), mixed well by vortexing, and then 10ml of Lysis Buffer (200mM NaOH and 1% SDS) added, and the tubes were turned upside down gently for a few times to achieve a homogeneous lysate without shearing the bacterial genomic DNA. Then 7.5ml of Neutralisation Buffer (3M KAc (pH5.5)) was added, mixed well, and centrifuged as before. The supernatant was filtered through 2 layers of gauze, 0.6 volume of isopropanol added, and the contents mixed by vigorous shaking before pelleting the plasmid DNA and bacterial RNA by centrifugation as before. The pellets were dissolved in 1xTE (see Section 7.7.1) containing 100μg/ml RNase and the mix was incubated at 37-55°C for 30min. At the end of the incubation, an equal volume of PEG solution (1.6M NaCl and 13% PEG) was added to precipitate plasmid DNA. The contents were mixed thoroughly by vigorous shaking, the DNA pellets recovered by centrifugation as before, and re-dissoved in 0.4ml 1xTE (see Section 7.7.1) by repeated pipeting. PCI extraction (see Section 7.13.1) was performed twice to get rid of PEG and DNA precipitated (see Section 7.9.1).

7.14. DNA sequencing.

PCR products were sequenced using cycle sequencing. They were sequenced on both strands using the same primers used in the initial PCR reaction. 3.2 pmols of primer was added to approximately 1μg of DNA and the total volume made up to 12μl with RQ grade ddH_2O. To this mixture was added 8μl of the Dyedeoxy Terminator Cycle Sequencing Prism Kit (Applied Biosystems). Reactions were carried out in a Perkin-Elmer 9600 thermal cycler using using 25 cycles 96°C for 15sec, 50°C for 1sec and 60°C for 4min or in a Perkin Elmer Thermal Cycler 480 using 25 cycles 96°C for 30sec, 50°C for 15sec and 60°C for 4min. The resultant DNA products were precipitated (see Section 7.9.1) and dissolved in 3-4μl of loading buffer (5x: 5 parts deionised formamide and 1 part 50mM EDTA containing 30mg/ml blue dextran). Running and analysis of sequencing gels was expertly performed by Robert MacFarlane (Beatson Institute, Glasgow, UK) as follows. Samples were run on a 6% polyacrylamide gel (made from a stock with 40% acrylamide and 2.1% bis-acrylamide) in 1xTBE buffer TBE (90mM Tris, 90mM boric acid and 2mM EDTA (pH8.0)) using an Applied Biosystem ABI 373A DNA Sequencer at 30W for 12h. Gels were analysed using 373A software version 1.2.1.

7.15. Northern blotting analysis.

7.15.1. Electrophoresis of RNA samples.

20μg of RNA was ethanol precipitated (see Section 7.9.1) and the pellets resuspended in 22μl of RNA sample buffer which contained the following:

2μl	5x formaldehyde gel-running buffer (5x FGRB: 0.1M MOPS (pH7.0), 40mM NaAc and 5mM EDTA (pH8.0))
3.5μl	formaldehyde (40.6%, v/v)
10μl	formamide (>99%)
2μl	10x formaldehyde gel-loading buffer (10x FGLB: 0.25% (w/v) bromophenol blue, 50% (v/v) glycerol and 1mM EDTA (pH8.0))
4.5μl	ddH_2O

The RNA samples, with 5μg of RNA size markers analysed in parallel, were denatured by incubation at 65°C for 15min followed by immediate chilling on ice. They were then loaded in 1.1% (w/v) agarose gel containing 6.5% (v/v) formaldehyde in 1xFGRB and electrophoresis was carried out in 1xFGRB (see above) at 80mM for 3-4h. At the end of electrophoresis, RNA gels were stained for 5min with an acridine orange dye solution

(15ug/ml in 3% (v/v) formaldehyde and 10mM Tris.HCl (pH6.8)) and then washed a few times in 1x FGRB (see above). 28s, 18s and low molecular weight RNA were visualised under a TF-35M UV luminator and this could give a rough idea with regard to the quality and loading of RNA samples.

7.15.2. Blotting of RNA gels.

Following electrophoresis, RNA gels were rinsed in dH_2O for 5min, followed by soaking in 20xSSC (3M NaCl, 0.6M sodium citrate, pH7.0) for 20-60min. Then they were transferred to Hybond™ N nylon membrane (Fingerprint grade) by capillary action overnight in 20xSSC. After transfer, the membrane was air-dried and UV cross-linked using a UV Stratalinker™ 1800 UV cross-linker. Lanes containing RNA markers were fixed in 0.5% acetic acid for 5-10min and then stained with 0.04% (w/v) methylene blue in 0.5M NaAc (pH5.2) for 5-10min.

7.15.3. Random-primed radiolabelling of DNA probes.

All DNA probes used for hybridisation to Northern blots were labelled with [α-^{32}P]dCTP using a random-priming kit (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, 100ng of probe in 8µl 1xTE (see Section 7.7.1) was denatured by boiling at 100°C for 5min and chilling on ice immediately. To the denatured probe, the following were added:

10x hexanucleotide mix	2µl
0.5mM dNTPs (dATP+dGTP+dTTP)	3µl
[α-^{32}P]dCTP (10µCi/µl)	5µl
Klenow enzyme (10U/µl, Gibco)	2µl

The reaction mix was incubated at 37°C for 30min. Unincorporated nucleotides were removed by gel filtration on a Sephadex G-50 column equilibrated in PBS (see Section 7.1.1). To prepared the columns, concentrated G-50 in PBS containing 0.02% (w/v) sodium azide was pipetted into the Disposable Chromatography Columns. The column was then attached to a microfuge tube and placed in a Falcon 15ml-tube which was then centrifuged at 1000rpm in a Sorvall Centrifuge RC3C using a H6000A/HBB6 swing-out rotor at 4-10°C for 6min to compress the column. The eluted PBS solution was discarded and the centrifugation process was repeated once more. Then the elutent was discarded and a fresh microfuge was attached to the column. Before purification of the probe, 80µl of 1xTE (see Section 7.7.1) was added to the above 20µl reaction mix after incubation at 37°C and the mixture was applied to the centre of the column and the radio-labelled probe was recovered by centrifugation as above. The eluent containing

the radio-labelled probe was boiled for 5min and then chilled immediately on ice to denature the probe.

7.15.4. Hybridisation of blots to the radiolabelled DNA probes.

The membranes were prehybridised in a 42°C shaking water bath for 1-3h or overnight depending on the strength of the signals expected. Hybridisation buffer contained 5xSSPE (20xSSPE: 3M NaCl, 0.2M NaH_2PO_4, 25mM EDTA, pH7.4), 50% (v/v) formamide, 5xDenharts (50xDenhardts: 1% (w/v) Ficoll-400, 1% (w/v) polyvinylpyrroli-done, 1% (w/v) BSA, 0.5% (w/v) SDS and 100μg/ml denatured salmon sperm DNA. Following prehybridisation, radio-labelled probe was boiled for 5min, chilled on ice and added to the hybridisation buffer. The membrane was subsequently hybridised overnight under the conditions described above. After hybridisation, the membranes were washed under increasingly stringent conditions as follows: twice in 2xSSPE, 0.1% (w/v) SDS at 20°C for 10min, once in 1xSSPE, 0.1% (w/v) SDS at at 20°C for 10min and finally 2-3 times in 0.1xSSPE, 0.1% (w/v) SDS at 65-68°C for 10-15min. The membranes were then exposed to a Kodak® X-OMAT®AR imaging film at -70°C for the required time.

7.15.5. Stripping blots of the radiolabelled probes.

The radio-labelled probe was stripped from the blots by placing in boiling 0.1% SDS and shaking until the solution cooled to room temperature. The blots were then hybridised to another probe as described in Section 7.15.4.

7.16. Scanning autoradiographs and quantitation of hybridisation signals by densitometry.

Autoradiographs were scanned by laser densitometer (Molecular Dynamics) and image analysis carried out using the PDQuest and Quantity 1 software from Protein Databases Inc.

7.17. Preparation of protein extracts.

7.17.1. Preparation of total protein extracts for Western blotting analysis.

Cells were grown to sub-confluence, harvested and washed thoroughly in ice-cold PBS. After removal of all the supernatant by aspiration, cell pellets were dispersed in ice-cold suspension buffer with freshly added proteinase inhibitors (100mM NaCl, 10mM Tris.HCl (pH7.6), 1mM EDTA (pH8.0), 1mM PMSF, 1μg/μl aprotinin, 1ug/ul leupeptin

and 5mM NaF). As soon as possible, an equal volume of 2x SDS gel-loading buffer (2x: 100mM Tris.HCl (pH6.8), 5% (w/v) SDS, 0.02% (w/v) bromophenol blue and 20% (v/v) glycerol) was added with freshly added β-mercaptoethanol (80µl per ml of 2x SDS gel-loading buffer). The samples were boiled for 5-10min and then, if necessary, sonicated twice with 5sec each time using a MSE Soniprep 150 ultra-sound sonicator. After sonication, samples were centrifuged in a microfuge for 10min at 4°C and the supernatant was transferred to fresh tubes. They were ready for use directly in SDS polyacrylamide gel electrophoresis (see Section 7.18.1)

7.17.2. Large scale preparation of nuclear protein extracts for gel retardation and DNA footprinting analysis.

The ELM-I/1 and I/2 cells were cultured at 10^5 per ml in 2 litre roller bottles containing 200-300ml medium that had been saturated with 5% (v/v) CO_2 air, and then placed on a New Brunswick RollaCell roller at 37°C. When the cell number had reached approximately 10^9, cells were pelleted by spinning at 1700 rpm (860g) for 10min at 4°C in a H6000A/HBB6 swing-out rotor using the Sorvall® centrifuge RC3C. The pellets were washed once with ice-cold PBS and resuspended in a hypotonic TMS solution (5mM Tris.HCl, 2.5mM $MgCl_2$ and 125mM sucrose, pH7.5) at a density of 1-2x10^7 cells per ml containing freshly added proteinase inhibitors (0.5mM benzamidine, 10mM β-glycerophosphate, 2mM levamisole, 0.5mM PMSF, 10mM sodium butyrate, 5mM sodium orthovanadate (pH8.0) and 1µg/ml each of aprotinin, bestatin, leupeptin and pepstatin). All the TMS solution used in the following steps contained all the proteinase inhibitors mentioned above. The suspension was centrifuged as above and the pellets resuspended in TMS/Triton (TMS with 0.05% (v/v) Triton-100) at a density of 2x10^7 per ml, to lyse the cytoplasmic membrane and release the nuclei. The nuclei were recovered by centrifugation at 800rpm (180-200g) for 5min at 4°C and washed 1-2 times with ice-cold TMS. The pellets were then resuspended in 50ml ice-cold TMS and 0.1ml of aliquot was used to check the amount of DNA in the nuclei preparation as follows. 0.1ml of the sample was mixed with 0.9ml of dH_2O and 9ml of 1M NaOH, the mixture was sonicated briefly to solubilise the DNA and the absorbance at 260nm was measured. The rest of the sample was centrifuged as above and the pellets resuspended in ice-cold TMS to give a the final DNA concentration of 5-10mg/ml. 1/10th volume of 4M NaCl was added dropwise into the sample on ice with stirring, followed by stirring on ice for another 10min and then centrifugation in a SS34 fixed-angled rotor using the Sorvall® RC-5B Superspeed Centrifuge at 17,000rpm for 15min at 4°C. The supernatant was transferred into 30ml ultracentrifuge tubes and ultracentrifuged in a T1270 rotor using Sorvall® OTD- ComBI Ultracentrifuge at 35,000rpm for 1h at 4°C. Then 0.45g of ammonium sulphate was added to per ml of the supernatant to precipitate

proteins. The mixture was vortexed to dissolve the salt and then stirred on ice for 30min. Proteins precipitates were recovered by spinning in a SS34 fixed-angled rotar using a Sorvall® RC-5B Superspeed Centrifuge at 15,000 rpm for 20 min at 4°C and the pellets dissolved in the minimum amount of a E50 solution (50mM $(NH_4)_2SO_4$, 5mM $MgCl_2$, 20mM HEPES (pH7.9), 0.1mM EDTA, 0.1% (w/v) Brij-35, 20% (v/v) glycerol) with freshly added 1mM DTT and proteinase inhibitors (see above). The samples were then dialysed overnight at 4°C in 1x storage buffer (50mM NaCl, 5mM $MgCl_2$, 20mM HEPES (pH7.9), 0.1mM $CaCl_2$, 0.1mM EDTA (pH8.0) and 20% glycerol) with freshly added 1mM DTT and proteinase inhibitors (see above). The next day, the samples were ultracentrifuged in a TLA100.3 rotor using the Beckman centrifuge TL100 at 35,000rpm for 1h at 4°C. The supernatant was aliquoted into sterile microfuge tubes and immediately frozen on dry ice.

For Western blot analysis (see Section 7.18), the appropriate amount of protein extract was mixed with 1x SDS loading buffer (see Section 7.17.1) and denatured by boiling for 5-10min. The samples were then ready for use in SDS polyacrylamide gel electrophoresis (see Section 7.18.1).

7.17.3. Quantitation of protein extracts.

The concentrations of protein extracts were determined by the Pierce BCA potein assay reagents following manufactures' instruction. The working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B (Reagents A and B were supplied by the company). 0.1ml of protein samples or each of the BSA protein standards was added to 2ml of the working reagent and the mixture was incubated at 60°C for 45min. After incubation, all samples were cooled to room temperature before the absorbance at 562nm was measured using a DU® 650 spectrophotometer.

7.18. Western blotting analysis.

7.18.1. SDS-polyacrylamide gel electrophoresis of protein samples.

Denatured protein samples in 1x SDS loading buffer (see Section 7.17.1 and 7.17.2) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the appropriate percentage (5-15%) of acrylamide resolving gels containing 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.04% TEMED in 376mM Tris.HCl (pH8.8). 5% acrylamide stacking gels, containing the same concentration of SDS, APS and TEMED, were made in 76mM Tris.HCl (pH6.8). Gels were prepared from a stock with 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide. Electrophoresis was carried out in 1x

protein-gel tank buffer (10x PGTB: 2M glycine, 1% (w/v) SDS and 250mM Tris.HCl (pH8.0)) at 150-200V.

7.18.2. Blotting of protein gels.

At the end of SDS-PAGE, protein gels were blotted onto ECL nitrocellullose membrane using a KEN EN TEC Semi Dry Protein Blotter according to manufacturer's instructions. Briefly, the transfer units were assembled as follows: (1) 6 layers of 3MM filter paper soaked in anode solution I (0.3M Tris, 20% (v/v) methanol, pH10.4) were placed on the anodic graphite plates; (2) on top of the 6 layers, 3 layers of 3MM filter paper soaked in anode solution II (25mM Tris, 20% (v/v) methanol, pH10.4) were placed; (3) these were followed by an ECL nitrocellulose membrane, pre-rinsed in dH_2O, and then the protein gel; (4) finally, 6 layers of 3MM filter paper that soaked in cathode solution (40mM 6-amino-n-hexanoic acid, 20% methanol, pH7.2) were placed on top of the protein gel. After each step of layering, bubbles were squeezed out gently using a glass rod. Finally, the transfer unit was covered with the cathode plate and the blotting was carried out at a constant current of $0.8mM/cm^2$ of gel for 1h at room temperature. Blots were then air dried and ready for the ECL detection of proteins of interest.

7.18.3. Detection of proteins using the ECL system.

The membranes were incubated in a blocking solution (5-10% (w/v) dairy milk powder in 1x Tris-buffered saline-Tween (1xTBS-T: 20mM Tris, 137mM NaCl and 3.8mM HCl, pH7.6, plus 0.1% (v/v) Tween 20)) at $4^{\circ}C$ overnight or at RT for 1-2h with constant vigorous shaking. After blocking, the blots were washed in 1xTBS-T (see above) as follows: two brief rinses at RT, one wash at RT for 15min with constant vigorous agitation and finally two washes at RT for 5min. Then the membranes were incubated with a 1:1-2,000 dilution of the primary antibody in TBS-T containing 5-10% (w/v) dairy milk power at RT for 1h or $4^{\circ}C$ overnight with constant vigorous agitation. At the end of the incubation, the membranes were washed as described above and then incubated with a 1:5,000 secondary antibody in TBS-T containing 5-10% (w/v) dairy milk power at RT for 1h with constant agitation. The membranes were then washed again as described above and antibody binding detected using the ECL system (Amersham). Equal volumes of detection solution I and II were mixed and the mixture placed onto the membranes with protein-side up After incubation for exactly 1min, the membranes were wrapped in Saran Wrap and exposed to a Fuji Medical X-ray imaging film for appropriate length of time.

7.18.4. Immuno-depletion of antibodies by the control antigen peptides.

The specificity of the primary antibody, rabbit-anti human *Erg* antibody, was tested by incubating the original antibody solution with 50µg of the *Erg* control peptides on ice for 30min prior to use in Wester blotting. The control peptides were the antigens that were used to generate the antibody.

7.19. *In vitro* transcription-translation.

In vitro transcription and translation of the *Fli-1* protein were carried using the TNT® T7 Quick Coupled Transcription/translation System (Promega) following the manufacture's instructions. The following reaction components were assembled:

TNT® T7 Quick Master Mix	40µl
[^{35}S]methionine (10µCi/µl, Amersham)	4µl
DNA template	1µg
nuclease-free ddH_2O	make up to 50µl

The reaction was incubated at 30°C for 90min. The radioactive translation products were analysed on an SDS-polyacrylamide gel as follows. 5µl aliquot was added to 20µl of 1x SDS-sample buffer (see Section 7.17.1). After denaturation by boiling at 100°C for 2min, the samples were then analysed by SDS-PAGE (see Section 7.18.1). At the end of the electrophoresis, the gel was dried down and exposed to a Kodak® X-OMAT ® AR imaging film for 6-16h at RT. For producing non-radioactive translation products, 1µl of 1mM methionine was substituted for the [^{35}S]methionine for gel retardation assays (see Section 7.20.3).

7.20. Gel retardation assays.

7.20.1. Preparation of double-stranded oligonucleotides.

250pmols of each complementry oligonucleotide were added to a solution containing 0.1M NaCl in 1xTE (see Section 7.7.1) in a total volume of 100µl. The mixture was boiled in a water bath for 10min and then left to slowly cool down to RT in the water bath. The concentration of the annealed double-stranded oligonucleotides was 2.5pmols/µl.

7.20.2. Preparation of probes.

5'-end labelling of double stranded oligonucleotides was carried out as previously described by Plumb *et al.* (1989). 2µl (5pmols) of double-stranded oligonucleotide annealed as described above was 5'-[^{32}P]-end labelled in the following reaction:

1µl	10x kinase buffer (Gibco)
0.5µl	100mM DTT
1µl	20mM spermidine (Sigma)
2.5µl	ddH_2O
2µl	[γ-^{32}P] dATP (10µCi/µl, Amersham)
1µl	T4 polynucleotide kinase (10U/µl, Gibco)

The above mixture was incubated at 37°C for 60min and then 68°C for 10min to inactivate the enzyme. Any ragged ends of the oligonucleotdes were then filled in by adding the following to the end-label reaction, followed by incubation on ice for 1h:

1µl	10x TA (0.33M Tris.Ac (pH7.9), 0.66 KAc, 0.1 $MgAc_2$, 5mM DTT and 1mg/ml BSA)
4µl	2.5mM dNTPs (Pharmacia)
4µl	ddH_2O
1µl	Klenow enzyme (1U/µl) (Gibco)

The oligonucleotides were then precipitated by adding 8µl of 1M NaCl, 1.5µl of 10mg/ml calf liver tRNA, 300µl of ethanol and cooling at -20°C for 1h. The mixture was spun for 15min in a microfuge and the pellet resuspended in 30µl of 1x DNA gel-loading buffer (see Section 7.11.1). The labelled oligonucleotides were then electrophoresed in a 1xTBE (see Section 7.14), 8% (w/v) polyacrylamide gel (prepared from a stock with 40% acrylamide and 2.1% bis-acrylamide at 100-150V for 1-2h in 1xTBE running buffer. The gel was exposed to a Kodak® X-OMAT®AR imaging film for 2min, the film developed and the radiolabelled oligonucleotides excised from the gel by aligning the autoradiograph with the gel. A second exposure was then taken to ensure the oligonucleotides had been removed. The gel slice was then incubated at 37°C overnight in 1ml 1xTE (see Section 7.7.1). To estimate the amount of unlabelled sequence used in competition experiments it was assumed that 80% of the oligonucleotides was recovered during the procudure, giving a concentration of labelled oligonucleotides of 4pmols/ml.

The sequence of the oligonucleotides used in gel retardation analysis are shown in Table 7.2.

Table 7.2. Oligonucleotides for gel retardation analysis.

Name	Sequence (Ets-binding sites were boxed)	Referecnes
E74	GATCTCTAGCTGAATAACCGGAAGTAACTC ATCCTAGGATC	Reddy and Rao, 1991.
mE74	GATCTCTAGCTGAATAACCCAAGTAACTCA TCCAGGATC	Reddy and Rao, 1991.
Ets3	ATACTCCAGAAGGAGGAAGCCAGTAGACAA	O'Prey *et al.*, 1993
Fli	TGAAACCGGAACTGGGC	Bosselut *et al.*, 1993
Pea3	GATCCTCGAGCAGGAAGTTCGAGATC	Wasylyk *et al.*, 1990.
PU1	GATCCATAACCTCTGAAAGAGGAACTTGGT TAGGTGATC	Klemsz *et al.*, 1990.
FP1	AAGTAGACTGATATTAACAAAGCTTAATA AATAATGTACCTCATG	
FP2L	ACCTCATGAAATAAAAAGCAGAAAGGAAT TTGAATAAAAATTTCCTGCATCTCATGC	
FP2S	AAGCAGAAAGGAATTTGAATAAAAATTTCC TGCATCTCATGC	
FP3	TCATGCCAACGGGGAAACACCAGAATCAAGT GTTCGGTGTAACTAAAGACACCCCTTCATCC A	
FP4	CCAAGAATGCAAAGCACATCCAATAAAAGA GCTGGATTATAACTATTCTTT	
FP5	GTCGGGACTTGAAGTGCCATTGGTACCTGC AGCTTC	
FP6	TTCTTTTCGGGGAAGGATGGCGCAAGCCGG GAGAAC	

7.20.3. DNA:protein binding reactions and gel electrophoresis.

The DNA:protein binding reaction was carried out in the following mix in a total volume of 15μl:

nuclear extract (0.1-5μg)
1μl of 1mg/ml poly(dI-dC) (Pharmacia)
un-labelled double-stranded competitors (5pmols for 250x) (when applicable)
1x storage buffer (see Section 7.17.2) with freshly added proteinase inhibitors
1mM PMSF and 1μg/μl aprotinin

The reaction mix was incubated on ice for 20-30min and then 5µl of labelled oligonucleotides (~20fmols) was added and the mix was incubated at 20°C for a further 30min. Samples were electrophoresed in a 5% (w/v) polyacrylamide 0.25xTBE (1xTBE see Section 7.14) gel (prepared from a stock with 40% acrylamide and 2.1% bis-acrylamide) for 2h at 150V at 4°C, after pre-running the gel for 1h under the same conditions. At the end of the electrophoresis, the gel was dried and exposed to a Koda® X-OMAT®AR imaging film.

7.20.4. "Supershift" analysis using antibodies.

In "supershift" analysis, 2µl of pre-immune serum or anti-serum were included in the DNA-protein binding mix prior to addition of probes. Incubation and gel electrophoresis were carried out as described above.

7.21. DNase I DNA footprinting analysis.

DNase I DNA footprinting analysis was carried out essentially as described by Plumb and Goodwin (1988).

7.21.1. Preparation of probes.

50µg of plasmid DNA was digested with an appropriate restriction enzyme in a total volume of 100µl for 2-3h (see Section 7.11.1). At the end of the the digestion, 20µl of calf intestinal alkaline phosphatase (1U/µl, Boehringer Mannheim) and the incubation at 37°C was continued for another 30min. Then, 10µl of 10% (w/v) SDS, 5µl of 2M NaCl and 5µl of EDTA (pH7.5) was added and the mixture heated at 68°C for 5min to inactivate the enzyme. The DNA was extracted with PCI and ethanol precipitated (see Section 7.13.1). The pellets were dissolved in 45µl of a solution containing 100mM Tris.HCl and 1mM EDTA (pH8.0). 7µl of such dephosphorylated DNA (approximately 7µg) was used for each 5'-end labelling reaction as follows.

The following reaction mix was set up:

dephosphorylated DNA (~1µg/µl)	7µl
10x Kinase Buffer (Gibco)	3µl
50mM DTT	3µl
20mM spermidine	3µl
ddH_2O	11µl

The mixture was heated at 70°C for 5min to denature the ends of the DNA and then quickly chilled at -20°C. It was then thawed on ice and 5µl of [γ-^{32}P]dATP (10µCi/µl), Amersham) and 0.7µl of T4 polynucleotide kinase (10U/µl, Gibco) were added and incubated at 37°C for 1h before adding 1µl of 0.2M EDTA (pH7.5) and incubation at 70°C for 10min to inactivate the enzyme. Then 100µl of TE (see Section 7.7.1) was added and the mixture deproteinised by PCI extraction (see Section 7.9.1). The aqueous phase was transferred to a fresh microfuge tube and 10µl of 4M NaCl, 3µl of calf liver tRNA (10mg/ml, Boehringer Mannheim) added, and the DNA was precipitated with 3 volumes of ethanol (see Section 7.9.1). The pellets were dissolved in 90µl of TE (see Section 7.7.1), 10µl of 1M KAc added and the DNA was re-precipitated with ethanol as above. The DNA pellets were then dissolved in 17µl of ddH_2O and the second restriction enzyme digest carried out in the appropriate buffer in a total volume of 20µl for 2h. At the end of the incubation, 5µl of 6x DNA gel-loading buffer (see Section 7.11.1) was added and the 5' labelled probe separated from the rest of the plasmid by agarose gel electrophoresis using a 1-1.5% agarose gel made from a stock of 40% acrylamide and 2.1% bis-acrylamide (see Section 7.11.2). The gel was exposed to a Kodak® X-OMAT®AR imaging film for 5min, the film developed and the radiolabelled oligonucleotides excised from the gel by aligning the autoradiograph with the gel. A second exposure was then taken to ensure the oligonucleotides had been removed. The probe was then purified using the Spin-Bind® DNA Recovery System for Agarose gel see section (7.11.3) and the DNA was eluted into 100µl of ddH_2O.

7.21.2. DNA:protein binding reactions.

The DNA:protein binding reaction mix, containing 100µg of nuclear protein extract, 6ug of poly(dI-dC) and 1-3ul of 5'-end labelled purified probe in a total volume of 100µl made up with 1xSB (see 7.17.2), was incubated on ice for 90min. In the control reaction, nuclear protein extract was replaced by 1xSB.

7.21.3. DNase I digestion of DNA-protein complexes.

The DNA:protein complex was then digested briefly with DNase I as follows. As DNA fragments were extremtely sensitive to DNase I, minor inconsistence in handling could cause large differences in the extent of digestion. To circumvent this problem: all handlings were kept as consistent as possible; the reactions were carried out one by one and, in addition; and various amounts of DNase I were used in each experiment. Briefly, 0.5µl, 1.0µl and 1.5µl of DNase I working dilution (~0.27µg/ml) was added to each sample, the mixture was vortexed rapidly and incubated at room temperature for exactly 30sec. At the end of the incubation, 100µl of footprint STOP buffer (82.4mM Tris.HCl, 8.2mM EDTA, 106mM NaCl, 1% (w/v) SDS, 66ug/ml calf liver tRNA and 0.4mg/ml

proteinase K, pH8.0) was added and the samples were uncubated at 37°C for 30min. The DNA was then denatured at 90°C for 2min and deproteinised by 2x PCI extraction (see Section 7.13.1) followed by 1x chloroform extraction (see Section 7.9.1). Then 15µl of 5M LiCl was added and the DNA was precipitated by the addition of 3 volumes of ethanol. The DNA was pelleted by spinning the samples at 14,000rpm in an Eppendorf microfuge and the pellets were washed once with ice-cold 75% (v/v) ethanol. After air-dry, the pellets were dissolved in 4-8µl of 1x Sequencing Gel Sample Buffer (0.025% (w/v) bromophenol Blue, 0.025% (w/v) Xylene Cyanol FF and 95% (v/v) formamide), denatured at 90°C for 3min and then immediately chilled at -50°C.

7.21.4. Sequencing-gel analysis of the digested probes.

DNase I digested and deproteinised DNA samples were separated in 6% denaturing polyacrylamide gels containing 42% (w/v) urea and the electrophoresis was carried out at 66 watts (~1600 Volts) for 2-3h at room temperature after pre-running the gels for 1h under the same conditions. The polyacrylamide gels were prepared from a stock solution with 40% acrylamide and 2.1% bis-acrylamide.

7.21.5. Localisation of footprints by A+G chemical sequencing.

The positions of footprints within the promoter were localised by utilising A+G chemical sequencing reactions (Papavassiliou, 1994). 9.5µl of ^{32}P-labelled probe were mixed with 0.5µl of calf liver tRNA (10mg/ml) and 1.5µl of 88% (v/v) formic acid. The reaction mix was incubated at 37°C for 14min, chilled on ice and 150µl of freshly prepared 1M aqueous piperidine added to cleave the DNA at 90°C for 30min. At the end of the incubation, the samples were chilled on ice and precipitated by the addition of 1ml of butan-1-ol followed by spinning at full speed in a microfuge for 2-5min. The pellets were resuspended in 1% SDS and reprecipitated with 1ml of butan-1-ol as above. The pellets were dried in a Heto Vac speedivac (Inter Med) for 5min, resuspended in 10-30µl of 1x Sequencing Gel Loading Buffer (see Section 7.21.6) and then separated in a denaturing polyacrylamide (see Section 7.21.6) gel along with samples from footprinting reactions.

CHAPTER 8: Materials

8.1. Cell lines, media and tissue culture supplies.

The ELM cell lines and all the MS-5 stromal feeder cell line were obtained from Dr. W. Ostertag in Heinrich-Pette Institute for Experimental Virology and Immunology, University of Hamburg, Germany. The K562, HL60, KG-1 and Molt-4 cells were taken from laboratory stocks. The quail Q2BN cells were kindly given by Dr. J. Frampton in Univeristy of Oxford, England. Recombinant human erythropoietin (Boehringer Mannheim) was the generous gift of Dr. T. Holyoake in the Western Infirmary, Glasgow.

Supplier: Gibco Europe Life Technologies Ltd., Paisley, Scotland
- Chicken serum
- Dulbeco's Modified Eagle's Medium (DMEM)
- α-Minimal Essential Medium (α-MEM)

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England
- Donor horse serum

Supplier: TCS Biologicals Ltd., Buckingham, UK
- Foetal calf serum

8.2. Mice.

Female C3H mice were purchased from Harlem-Olac and housed by S. Bell and injected by T. Hamilton from the Beatson Animal Laboratory Services. The Beatson Institute's Alycon II Teletherapy Unit was used for all irradiation procedures.

8.3. Bacterial hosts and media.

E.coli host strain DH5α was obtained from laboratory stock held by Fiona McGreger. Terrific broth and L-broth were prepared according to Sambrook *et al.* (1989).

8.4. Nucleotides, polynucleotides, DNA, DNA/RNA markers.

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England
- [α-^{32}P]dCTP ~3000Ci/mmol
- [γ-^{32}P]dATP ~5000Ci/mmol

L-[^{35}S] methionine >1000Ci/mmol

Supplier: Bethesda Research Laboratory, Gibco Ltd., Paisley, Scotland

DNA markers (1μg/μl): 1kb ladder and bacteriophage Φx174DNA (Hae III-cut)

0.24-9.5kb RNA markers (1μg/μl)

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England

Calf liver tRNA

Supplier: Pharmacia Ltd., Milton Keynes, Buckinghamshire, England

Poly (dI-dC)

Ultrapure dNTP Set (100mM)

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England

Sonicated and denatured salmon testes DNA (10mg/ml)

8.5. Plasmids.

Plasmid	Suppliers
pCR-TA	Invitrogen Corporation, San Diego, California, USA
pCR-Scrip™.Amp.SK(+)	Stratagene Ltd. UK, Cambridge, England
α-*globin*	Dr. R. Nibbs (Beatson Institute)
pSSFV(*neo-bcl-2*)	Dr. S. Korsmeyer, Washington University, Missouri, USA
7s rRNA	Dr. L. Wu (Beatson Institute)
pRC-CMV(*erg*)	Dr. R. Nibbs (Beatson Institute)
pRC-CMV(*fli-1*)	Dr. C. Bartholomew (Beatson Institute)
pRC-CMV(DNM1)	Dr. C. Bartholomew (Beatson Institute)
pRC-CMV(DNM2)	Dr. C. Bartholomew (Beatson Institute)
bcl-x	Dr. J. Reed, La Jolla Cancer Research Foundation, USA
scl	Dr. A.R. Green, University of Cambridge, England
pXP2 (h250)	Dr. J. Frampton, University of Oxford, England
pXP2 (h600)	Dr. J. Frampton, University of Oxford, England
pGL2 (basic)	Dr. C. Bartholomew (Beatson Institute)
pGL2 (min. tk)	Dr. C. Bartholomew (Beatson Institute)
pGL2 (3xE74+min.tk)	Dr. C. Bartholomew (Beatson Institute)
pGK (β-*gal*)	Dr. J. Frampton, University of Oxford, England
pHSV (β-*gal*)	J. O'Prey (Beatson Institute)
pRXV (β-*gal*)	Dr. J. Frampton, University of Oxford, England

8.6. Enzymes and enzyme inhibitors.

Supplier: Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland

All restriction endonucleases with 10x reaction buffers
DNase I (2mg/162µl)
Proteinase K
T4 DNA ligase (1U/µl) and 5x ligation buffer
Taq DNA polymerase (10U/µl) and 10x PCR reaction buffer

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England

Calf intestinal alkaline phosphatase (1U/µl)
DNase-free RNases
RNases A

Supplier: Northumbria Biologicals Ltd. (NBL), Cramlington, Northumberland, England

Klenow DNA polymerase (1U/µl)
T4 polynucleotide kinase (10U/µl) and 10x kinase buffer

Supplier: Sigma Chemical Co. Ltd., Poole, Dorset, England

Aprotinin
Benzamidine
Bestatin
Diethylpyrocarbonate (DEPC)
β-glycerophosphate
Levamisole
Leupeptin
Pepstatin A
PMSF
Sodium butyrate
Sodium orthovanadate

Supplier: Stratagene Ltd. UK, Cambridge, England

Pfu DNA polymerase (2.5U/µl) and 10x reaction buffer

8.7. Antibodies and control peptides

Rabbit-anti-mouse *Fli-1* anti-serum was a kind gift from Dr. C. Bartholomew.

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England

Rabbit IgG, horseradish peroxidase-linked whole antibody (donkey)

Supplier: PharMigen Deutschland GmbH, Hamburg, Germany

Rabbit-anti-mouse *Bcl-2* anti-serum

Supplier: Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA

Purified polyclonal rabbit-anti-human *Erg* antibody (100μg/ml)

Bcl-2 control peptides (100μg/0.5ml)

Erg control peptides (100μg/0.5ml)

8.8. Molecular biology kits.

Supplier: Amersham International plc, Little Chalfont, Buckinghamshire, England

ECL™ Western Blotting Analysis System

Suppler: Applied Biosystems, Warrington, UK

Dyedeoxy Terminator Cycle Sequencing Prism Kit

Supplier: Boehringer Mannheim UK, Lewes, East Sussex, England

Random-primed DNA labelling kit

Supplier: FMC BioProducts Europe, Vallensbaek Strand, Denmark

SpinBind® DNA recovery system for agarose gels

Supplier: Invitrogen Corporation, San Diego, California, USA

TA cloning kit

Supplier: Promega, Madison, Wisconsin, USA

TNT® T7 Quick Coupled Transcription/Translation System

Luciferase assay system

Supplier: Stratagene Ltd. UK, Cambridge, England

PCR-Script™.Amp.SK(+) cloning kit

8.9. Membranes.

Supplier: Amersham International plc., Little Chalfont, Buckinghamshire, England

Hybond™ N nylon membrane (fingerprint grade)

ECL™ nitrocellulose membrane

8.10. Chemicals and water.

Supplier: Bethesda Research Laboratory, Gibco Ltd., Paisley, Scotland
TRIzol reagent

Distilled water for buffers and general solutions was obtained from a Millipore MilliRO 15 system. Water for protein/enzyme work or recombinant DNA protocols was further purified on a Millipore MilliQ system to 18MΩcm.

8.11. Equipment.

Suppler: Applied Biosystems, Warrington, UK
ABI 373A DNA Sequencer

Supplier: BioRad Laboratories, Richmond, California, USA
BioRad Gene Pulser™ with capacitance extender

Supplier: Eppendorf, Hamburg, Germany
Eppendorf Multipette® 4780

Supplier: Flowgen Instruments, Ltd., Lichfield, Staffordshird, UK
EasyjecT Plus

PART III: RESULTS

CHAPTER 9: Cellular and Molecular Events in ELM-D Cells upon stroma-withdrawal

9.1. Viability assay of ELM-D cells upon stroma-withdrawal.

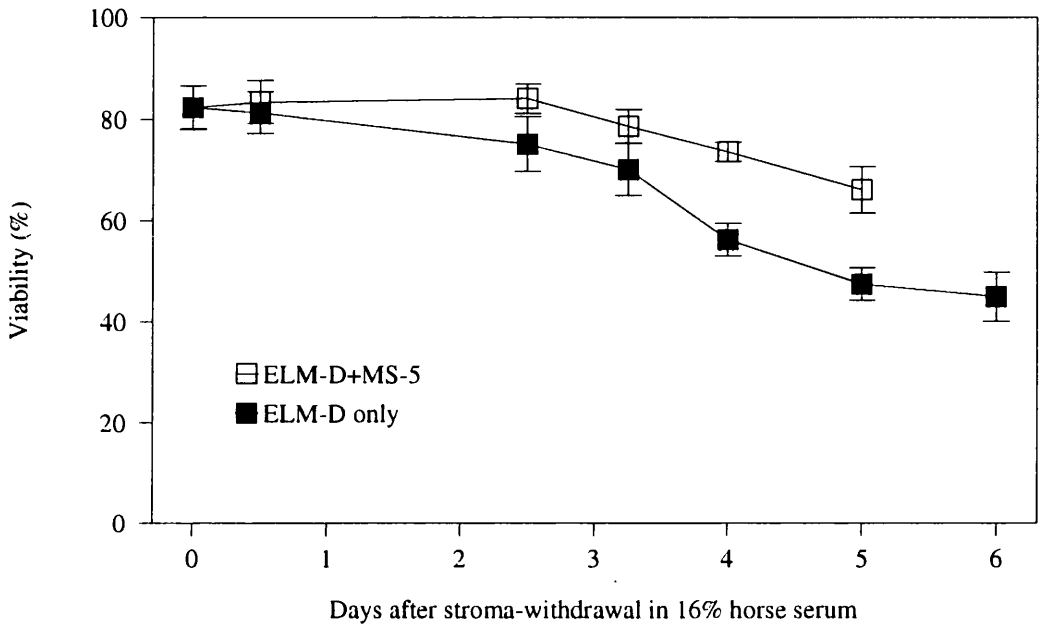
As mentioned in Chapter 6, one of the unusual features of the ELM system is that the erythroleukaemia cells growing *in vivo* in the spleen cannot be maintained *in vitro* except in contact with bone marrow-derived stromal cells. The stroma-dependence of the cell line (ELM-D) derived from the primary tumour has been rigorously tested by serial re-cloning experiments in the presence or absence of stromal cells (Prof. W. Ostertag, personal communication). The results confirmed that ELM-D cells show a strict dependence on stromal cells for long-term growth and survival, but are capable of proliferative cell expansion for a short term without stroma (2-3 weeks). However, their experiments also indicate that the ELM-D cell population contains a small proportion of cells that can grow without stromal cells and the frequency of occurence of stroma-independent clones (ELM-Is) is approximately 10^{-5}.

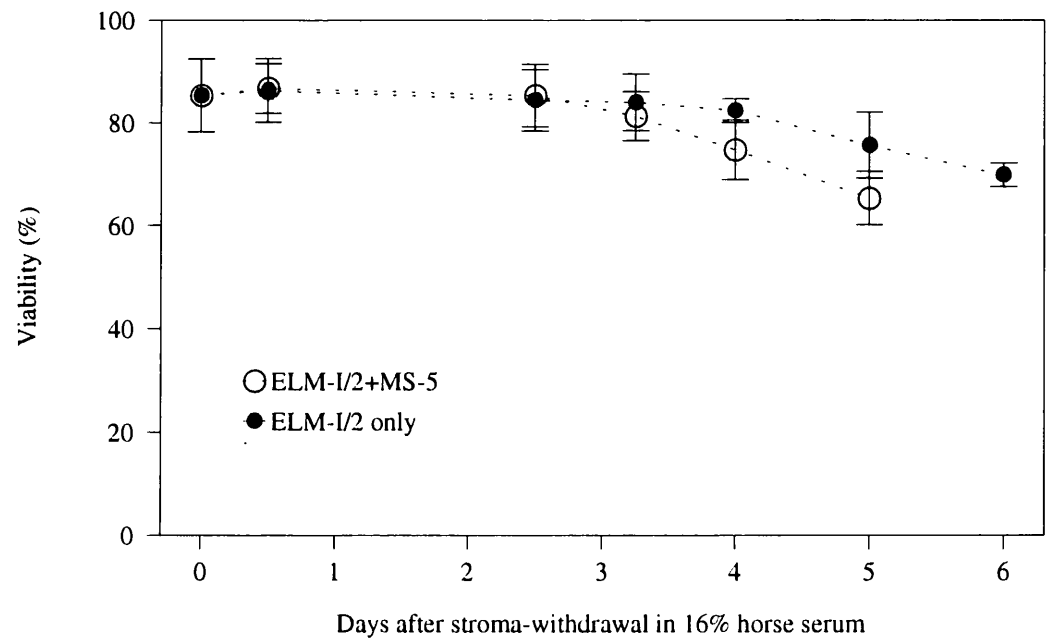
As large number of cells are required for more detailed cellular and molecular characterisation, the stroma-dependence of ELM-D cells has to be reproduced in mass culture experiments. In order to do so, ELM-D cells were cultured in the presence of stromal cells in the ELM growth medium containing 16% horse serum (see Methods and Materials). They were grown to confluence when they were shaken off from the stromal cells. They were then centrifuged and the pellet resuspended in fresh medium at a density of 10^5 per ml and re-seeded into flasks with or without stromal cells. To eliminate the stromal cell contamination which would affect the viability of ELM-D cells in stroma-withdrawal experiments, the cell suspension was transferred to a new flask every hour for 3-4h. In this way, the adherent stromal cells were eliminated, whereas ELM cells, which are suspension cells, only loosely attached to the bottom of the flask and were lifted up by gentle agitation of the medium. It has been confirmed that, in this way, the stromal cell contamination can be eliminated almost completely. As a control, the stroma-independent ELM-I/2 cells were also grown on the stromal cells and subject to the same manipulation as ELM-D cells. The viability of ELM-D and ELM-I/2 cells in the presence or absence of the stromal cells was measured by the trypn blue exclusion method (see Methods and Materials) every day over a week period.

It can be seen from Figure 9.1 that the viability of ELM-D cells in the presence of the stromal cells (open square) remained constant up to day 3, after which it declined slightly, presumably due to confluence of the culture. On the contrary, the viability of ELM-D cells deprived of the stromal cells (solid square) decreased gradually over the

Figure 9.1. Viability assays of ELM-D cells upon stroma-withdrawal.

The ELM-D cells and ELM-I/2 cells were cultured to sub-confluence on the stroma in the ELM growth medium containing 16% serum. They were then shaken off from the stroma and re-seeded into flasks with or without stromal cells. The viability of ELM-D and I/2 cells in the presence or absence of the stroma was measured by the trypan blue exclusion method (see Methods and Materials). Each experiment was carried out in triplicates, and the graph shows the means and standard deviation of two independent experiments.





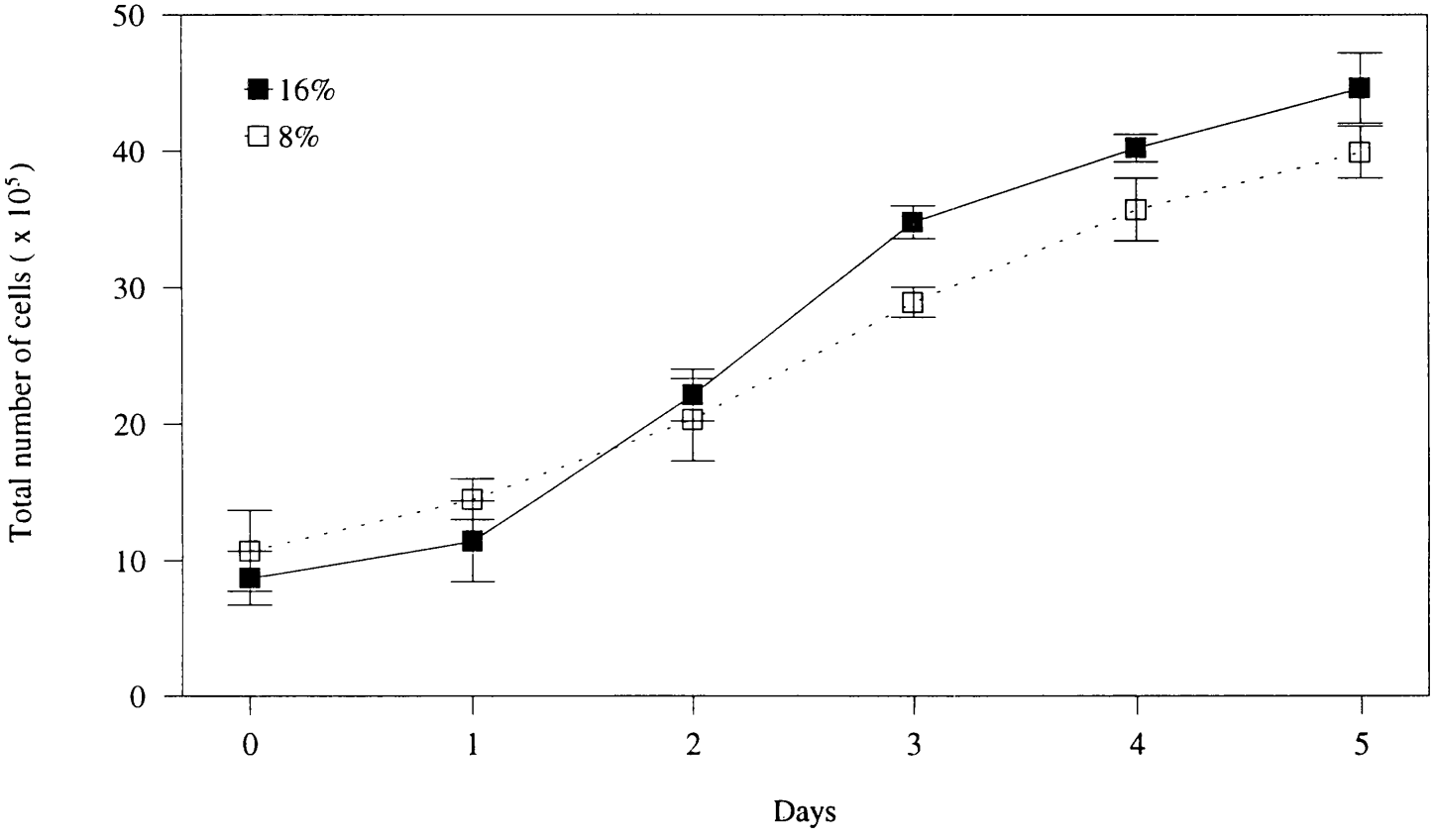
period of experiments. The viability dropped to about 50% at day 6. The viability of ELM-I/2 cells in the presence (open circle) or absence (solid circle) of stromal cells was similar to that of ELM-D cells in the presence of stroma, indicating that the decline in viability observed in ELM-D cells in the absence of stroma was not due to experimental manipulation. When the culture of ELM-D cells in the absence of the stroma was maintained continuously, it was found to be able to be grow for an extended period of time.

The fact that only 50% of ELM-D cells die a week after stroma-withdrawal and the whole culture can never die out does not necessarily mean that our data is contradictory to that of the serial re-cloning experiments and overthrows the established fact that ELM-D cells are stromal dependent. It should bear in mind that the growth conditions in serial re-cloning experiments is much more stringent than that in the mass culture. Moreover, given that ELM-D cells are fairly heterogenous, and a small number of clones have been derived in the absence of stromal cells even in serial re-cloning experiments (Prof. W. Ostertag, personal communication), it is possible that the small number of stroma-independent cells present in the ELM-D cell population can proliferate rapidly, and sooner or later will take over in the mass culture. In addition, it is also possible that the strictly stroma-dependent ELM-D cells die only slowly in the absence of the stromal cells and can still proliferate, to some extent, over a limited period under the culture condition. This is reminiscent of the fact that, in the serial re-cloning experiments, there are clones of ELM-D cells grown up in the absence of the stroma, which can grow for 2-3 weeks yet disintegrate afterwards.

In order to establish the culture conditions under which growth of ELM-D cells is more stringently stroma-dependent, attempts were made to culture ELM-D cells in the reduced levels of serum. Serum contains various survival and proliferation that might keep ELM-D cells alive and stimulate proliferation in the absence of stromal cells. The ELM-D cells were grown in the medium containing 16%, 8% or 4% horse serum. By visual observation of the cultures under the light microscope, it was noted that ELM-D cells grown in 16% and 8% serum could grow and reach confluence at a similar rate. Measurement of the numbers of ELM-D cells maintained in 16% and 8% serum further confirmed that cells proliferate similarly at both serum conditions (Figure 9.2). However, cells maintained in 4% serum grew at a much reduced rate and still remained pretty sparse when the stromal layer reached confluence at 4-5 days. Regardless of the issue if ELM-D cells grown at 4% serum were healthy and could be mamintained for a long term, they fact that they grew too slow eliminated the possibility of growing cells at this level of serum.

Figure 9.2. Growth curves of ELM-D cells in the presence of stroma in 16% and 8% serum.

The ELM-D cells were cultured to sub-confluence on the stroma in the ELM growth medium containing 16% or 8% serum. They were then shaken off from the stroma and counted using a haemocytometer at the time points indicated. Each experiment was carried out in triplicates, and the graph shows the means and standard deviation of two independent experiments.



Similar viability assays were performed in the stroma-withdrawal experiments when 8% serum was used as the culture condition. Figure 9.3 shows that the viability of ELM-D cells decreased dramatically upon stroma-withdrawal (solid square) and it dropped to approximately 10% at day 6, whereas ELM-D cells grown on the stroma (open square) maintained the high viability. The control experiments showed that the viability of ELM-I/2 cells either in the presence or absence of stromal cells remained constantly high over the period of the assays. Thus, using 8% horse serum as the culture condition largely reduces the viability of ELM-D cells upon stroma-withdrawal.

9.2. Characterisation of the growth of ELM-D cells in the absence of stromal cells in 8% horse serum.

To further characterise the growth of ELM-D cells in the absence of the stroma, similar stroma-withdrawal experiments were carried out in 8% serum, but the actual number of viable cells instead of viability was measured. It can be seen from Figure 9.4 (a and b) that, upon stroma-withdrawal, the total number of viable ELM-D cells decreased dramatically. It dropped to approximately 1/10th of the original number at day 3 and 1/100th at day 7. The number of viable cells remained relatively constant for the following 9 days, after which it increased logarithmically (Figure 9.4b). Calculations of the total number of viable cells have taken account of the dilutions of cells during passage.

The timing and extent of this later outgrowth of stroma-independent cells were dependent on the level of serum. At day 8 after the removal of stroma, half of the cells was passaged into medium containing 16% horse serum. It can be seen that the outgrowth of the stroma-independent cells in 16% serum (dotted line) started at day 12 instead of day 17. Furthermore, the proliferation rate of those cells in 16% serum was at least twice as much as that in 8% serum.

The above evidence further confirms that, in 8% serum, ELM-D cells are truly stroma-dependent, and majority of the population dies out following stroma-withdrawal. However, 16% serum allows a high background of stroma-independent growth.

9.3. Effects of serum levels on the long-term growth of ELM-D cells on the stroma.

It is critical that cells grown in 8% serum can not only proliferate at a similar rate but also maintain their biological features. The most important feature of ELM-D cells is that they are stroma-dependent and the stromal cells are able to maintain their survival and growth for a long term. Therefore, it is crucial to test whether the reduced level of serum affects the ability of stromal cells to maintain the long-term growth of ELM-D

Figure 9.3. Viability assays of ELM-D cells upon stroma-withdrawal in 8% serum.

The ELM-D and ELM-I/2 cells were cultured to sub-confluence on the stroma in the ELM growth medium containing 8% horse serum. They were then shaken off from the stroma and re-seeded into flasks with or without stromal cells. The viability of ELM-D and I/2 cells in the presence or absence of the stroma was measured by the trypan blue exclusion method (see Methods and Materials). The graph shows the means and standard deviation of two independent experiments, each carried out in triplicates

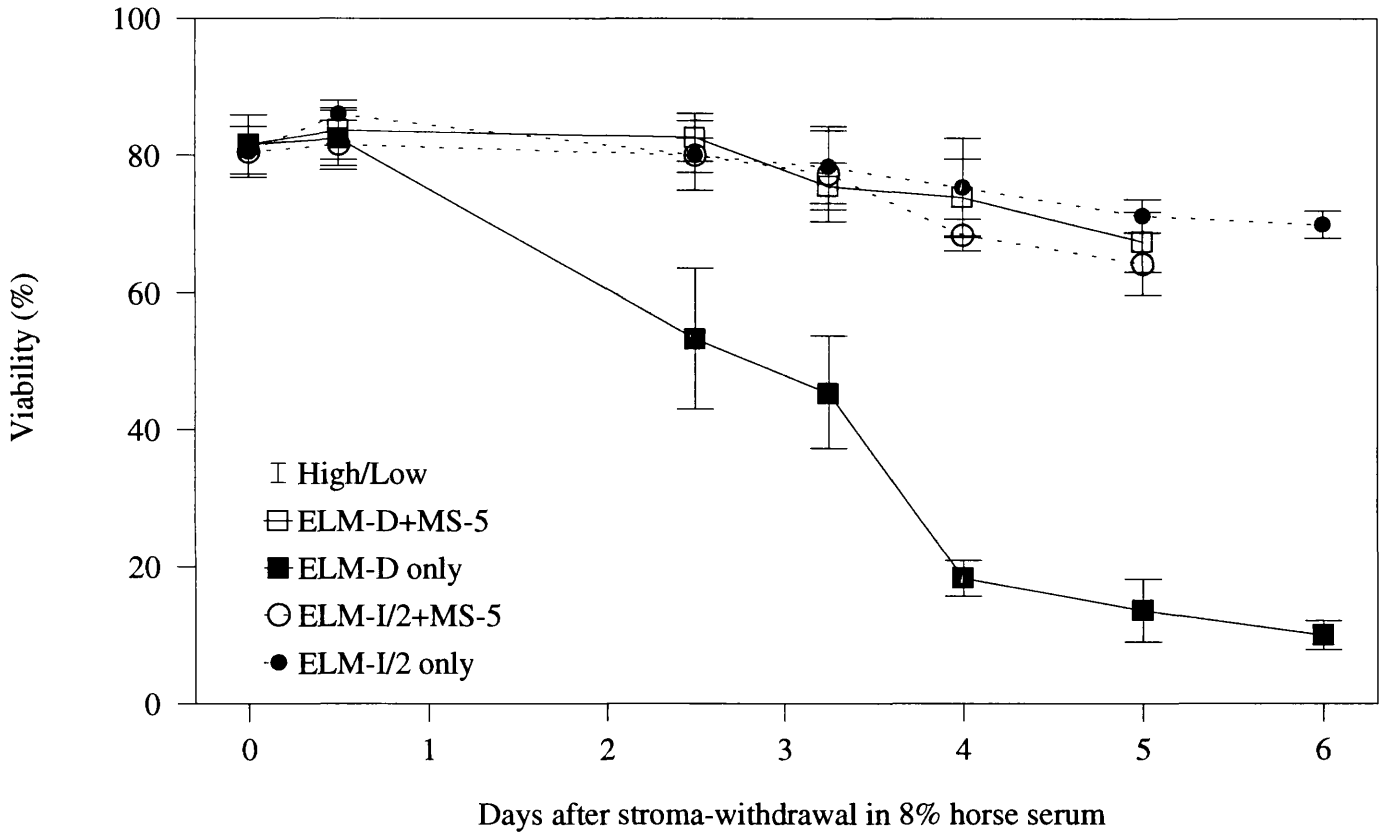
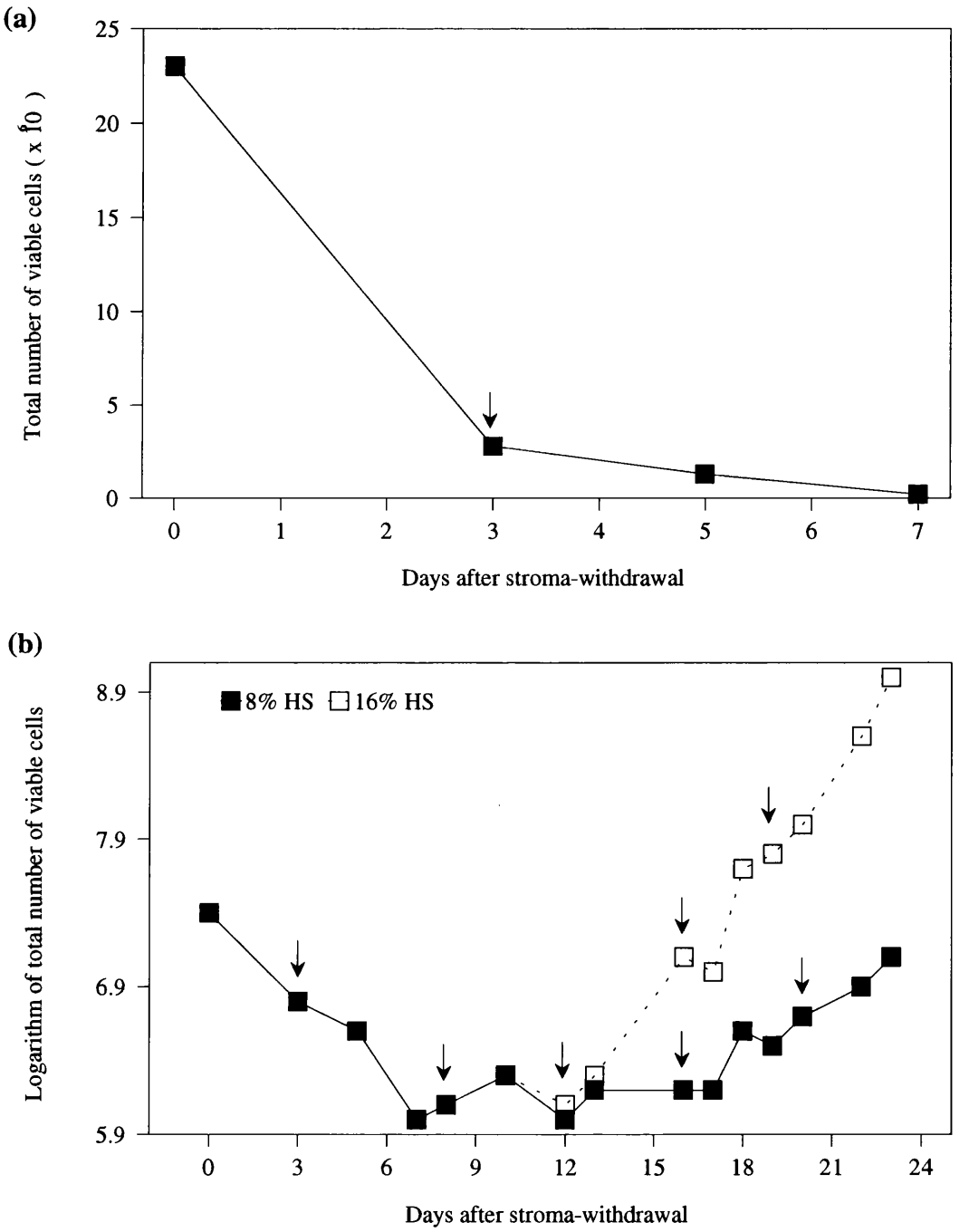


Figure 9.4. Characterisation of the growth of ELM-D cells in the absence of stroma in 8% serum.

The ELM-D cells were cultured to sub-confluence on the stroma in the ELM growth medium containing 8% serum. They were then shaken off from the stroma and re-seeded into flasks with or without stromal cells. The viability of ELM-D cells in the presence or absence of the stroma was measured by the trypan blue exclusion method (see Methods and Materials). Total number of viable cells was calculated taking account of the dilution of cells during each passage. At day 8 after the removal of stroma, half of the cells was passaged into medium containing 16% horse serum. The dotted line represents the total number of viable cells (in logarithm) maintained in 16% serum. Arrows indicate when cells were passaged.



cells. For this purpose, serial re-cloning experiments were performed. The ELM-D cells were grown to sub-confluence on the stroma in the ELM growth medium containing 16% or 8% horse serum. They were then shaken off from the stroma and cloned by end-point dilution into 96-well plates containing a monolayer of stromal cells. After every 2-3 weeks, the number of clones grown out were counted, and 8-16 clones were picked and subsequently re-cloned. Table 9.1 shows the results of four rounds of such cloning experiments which lasted for 3 months.

Table 9.1. Comparison of the cloning efficiency (C.E.) and colony size of ELM-D cells cloned in the presence of stromal cells in serial re-cloning experiments in 16% and 8% serum. Colony size represents the total number of cells within each colony and is an average of 10 randomly picked colonies. After each round of cloning, colonies were counted and cloning efficiency calculated; then 10 colonies were picked randomly, the total number of cells within each colony counted using a haemocytometer, and subsequently re-cloned. The data of the first three rounds of cloning experiments was a combination of three independent experiments. The fourth round of cloning was only performed in one occasion due to the large amount of work involved.

Serum level		16%		8%	
		C.E.	Size (x 10^3)	C.E.	Size (x 10^3)
1st round	Mean	40.5%	n.d.	35.6%	n.d.
	S.D.	9.8%	n.d.	13.2%	n.d.
2nd round	Mean	15.9%	30.4	18.9	21.8
	S.D.	10.1%	17.6	9.3%	14.1
3rd round	Mean	8.3%	24.7	8.9%	15.9
	S.D.	5.1%	14.6	6.4%	10.0
4th round	Mean	15.6%	34.4	14.0%	30.8
	S.D.	10.7%	14.4	11.2%	14.5

It can be seen that the cloning of ELM-D cells on the stroma, in terms of both cloning efficiency and colony size, was not significantly different under different serum conditions used. More importantly, apart from the second round of cloning (see below), there was no decline in cloning efficiency of ELM-D cells in 8% serum over the period of experiments (3 months). This suggests that ELM-D cells are able to grow in a long term on the stroma in 8% horse serum.

The cloning efficiency of ELM-D cells in the first round of cloning was much higher than subsequent rounds. It should be noted that the first round of cloning involved cells maintained in a mass culture, which are generally cloned better than cloned cells. Another feature of such serial re-cloning experiments, which is also reflected by the data

shown in the table, is the large variation in terms of both cloning efficiency and colony size between different clones. The only way to get around this problem so as to obtain statistically significant results is to select a large number of clones and repeat the experiment several time, as was done in the experiments presented.

Thus, the lowered serum level (i.e. 8%) does not affect the long-term maintenance of ELM-D cells by the stroma and, therefore, can be used as the culture condition for further cellular and molecular characterisation.

9.4. Morphological studies of ELM-D cells upon stroma-withdrawal.

To characterise the features of the cell death that ELM-D cells undergo upon stroma-withdrawal, Giemsa staining and microscopy analysis were performed (see Methods and Materials). Briefly, ELM-D cells were grown in the presence of stroma to sub-confluence in 8% serum, and were then shaken off from the stroma and re-seeded into flasks without the stromal cells. Four days later, cells were harvested and centrifuged onto microscope slides using a cytospin centrifuge, and stained with a Giemsa solution. Cells that had just been shaken off from the stroma were also stained for comparison.

The ELM-D cells just shaken off from the stroma displayed typical morphology of pro-erythroblasts or basophilic erythroblasts with some differentiation to orthorchromatic erythroblasts (Harrison *et al.*, 1973) (Figure 9.5a). However, it can be seem from Figure 9.5b showed that ELM-D cells removed from the stroma for 4 days displayed membrane blebs, cytoplasmic vacules, chromosome condensation and classical apoptotic bodies (indicated by arrows). This suggests that, as a result of stroma-withdrawal, ELM-D cells die with the characteristics of apoptosis. This has been confirmed by our collaborator, Prof. W. Ostertag, by using "TUNEL" assays, which showed that ELM-D cells removed from stroma exhibit DNA strand breaks, which is the most prominent feature of apoptosis (Savill *et al.*, 1993).

9.5. Changes in gene expression of ELM-D cells upon stroma-withdrawal.

For most cell types, the above features observed, in particular, the existence of apoptotic bodies and DNA strand breaks, are sufficient for diagnosis of apoptosis. However, the situation is not so simple for erythroid cells since chromosome condensation and DNA strand breaks also appear in cells that undergoing erythroid differentiation. During this process, the nucleus and the associated chromosomes are condensed, ultimately degraded and exocytosed out of the cell (Harrison *et al.*, 1973). It has also been reported that DNA isolated from differentiating erythroid cells also appears in a pattern of laddering in agarose gel electrophoresis (Savill *et al.*, 1993), which is normally observed

Figure 9.5. Giemsa staining of ELM-D cells after stroma-withdrawal.

(a) ELM-D cells that had just been shaken off from the stromal cells;
(b) ELM-D cells that had been removed from the stroma for 4 days.
(Scale bar = 10μm)

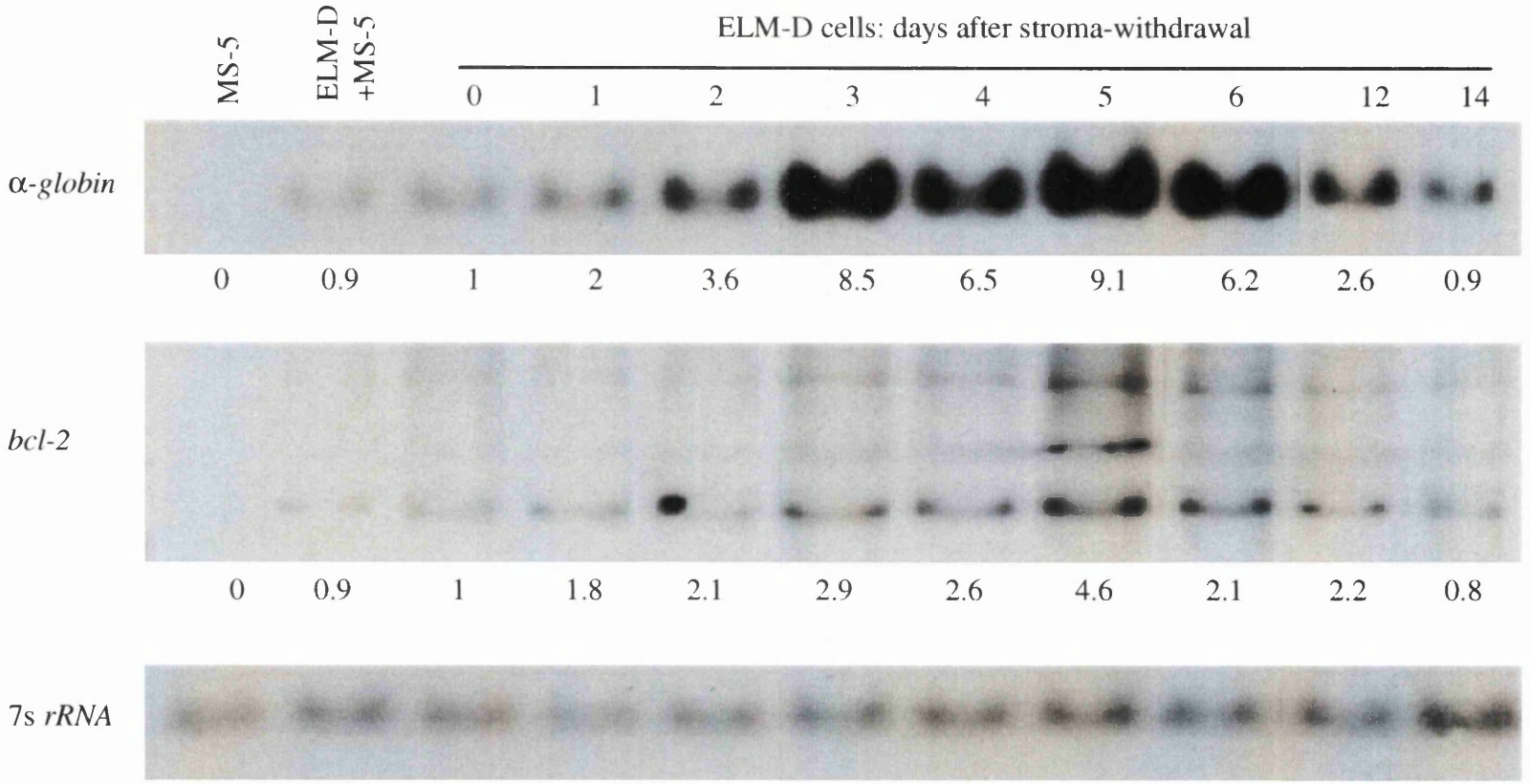
in apoptotic cells. Therefore, to define the nature of the cell death of ELM-D cells upon stroma-withdrawal, it is necessary to perform experiments to distinguish whether apoptosis or erythroid differentiation is in fact occurring. In order to do so, the *α-globin* and *bcl-2* expression was analysed as markers for erythroid differentiation and apoptosis, respectively. As mentioned in Chapter 5, *bcl-2* is the founding member of an expanding family of genes that are involved in the regulation of apoptosis. It is able to protect cells from apoptosis during development *in vivo* and in many culture systems *in vitro*, and has been found to be up-regulated as a cellular response to apoptosis stimuli (Vaux *et al.*, 1988). Briefly, ELM-D cells grown on stroma were shaken off from the stroma and transferred at 10^5 cells per ml to flasks containing no stromal cells. Any contamination of the stromal cells was eliminated as described previously (see Section 9.1). Cells were harvested at various time points after stroma-withdrawal, total RNA was extracted and analysed by Northern blotting (see Methods and Materials). The same blot was successively probed with a mouse *bcl-2* cDNA fragment and *α-globin* genomic fragment (spanning exon 1 and 2 of the gene). The RNA loadings were assessed by stripping the blot and re-probing it with a *7s rRNA* genomic fragment.

9.5.1. Changes in the *α-globin* and *bcl-2* mRNA expression in ELM-D cells upon stroma-withdrawal in 16% serum.

Figure 9.6 shows the changes in *α-globin* and *bcl-2* expression of ELM-D cells upon stroma-withdrawal in 16% serum. It can be seen that ELM-D cells deprived of the stroma displayed a gradual increase in *α-globin* expression, which peaked at day 5 and reached approximately 9 fold as the original level, indicating that ELM-D cells underwent erythroid differentiation upon stroma-withdrawal. Thereafter, the expression of *α-globin* decreased to about the same as the control level at 14 days after stroma-withdrawal. The occurrence of spontaneous erythroid differentiation in ELM-D cells after the stroma was removed implies that the stroma is able to block an intrinsic differentiation program within, at least, a sub-population of the cells. This can be one of the mechanisms whereby the stroma maintains the long-term survival and growth of ELM-D cells. The later decline of the *α-globin* expression within the population may be a result of the outgrowth of another sub-population of the cells which is stroma-independent and does not differentiate in the absence of it. Cells that underwent terminal erythroid differentiation would ultimately die and be eliminated from the population. On the other hand, those that did not differentiate and therefore expressed low levels of *α-globin* would grow out. As a consequence, the overall *α-globin* expression would decrease eventually.

In addition, the expression of *bcl-2* also displayed an early increase, which peaked at day 5 to about 5 fold as the original level, and a later decline. The significance and

Figure 9.6. Changes in the α-*globin* and *bcl-2* mRNA expression in ELM-D cells after stroma-withdrawal in 16% serum by Northern blotting analysis. The figures below the lanes are the relative levels of gene expression after calibration with the *7s rRNA* loading control.



implication of the changes in *bcl-2* expression are difficult to interpret. It is possible that the increase in *bcl-2* expression was due to the deprivation of various survival and proliferation signals that are normally provided by the stromal cells. Alternatively, since the *bcl-2* expression increased and decreased in parallel with that of *α-globin*, it is possible that the *Bcl-2* protein may positively regulate erythroid differentiation. To distinguish these possibilities, ELM-D cells should be induced to differentiate in the presence of the stromal cells (also see Chapter 12).

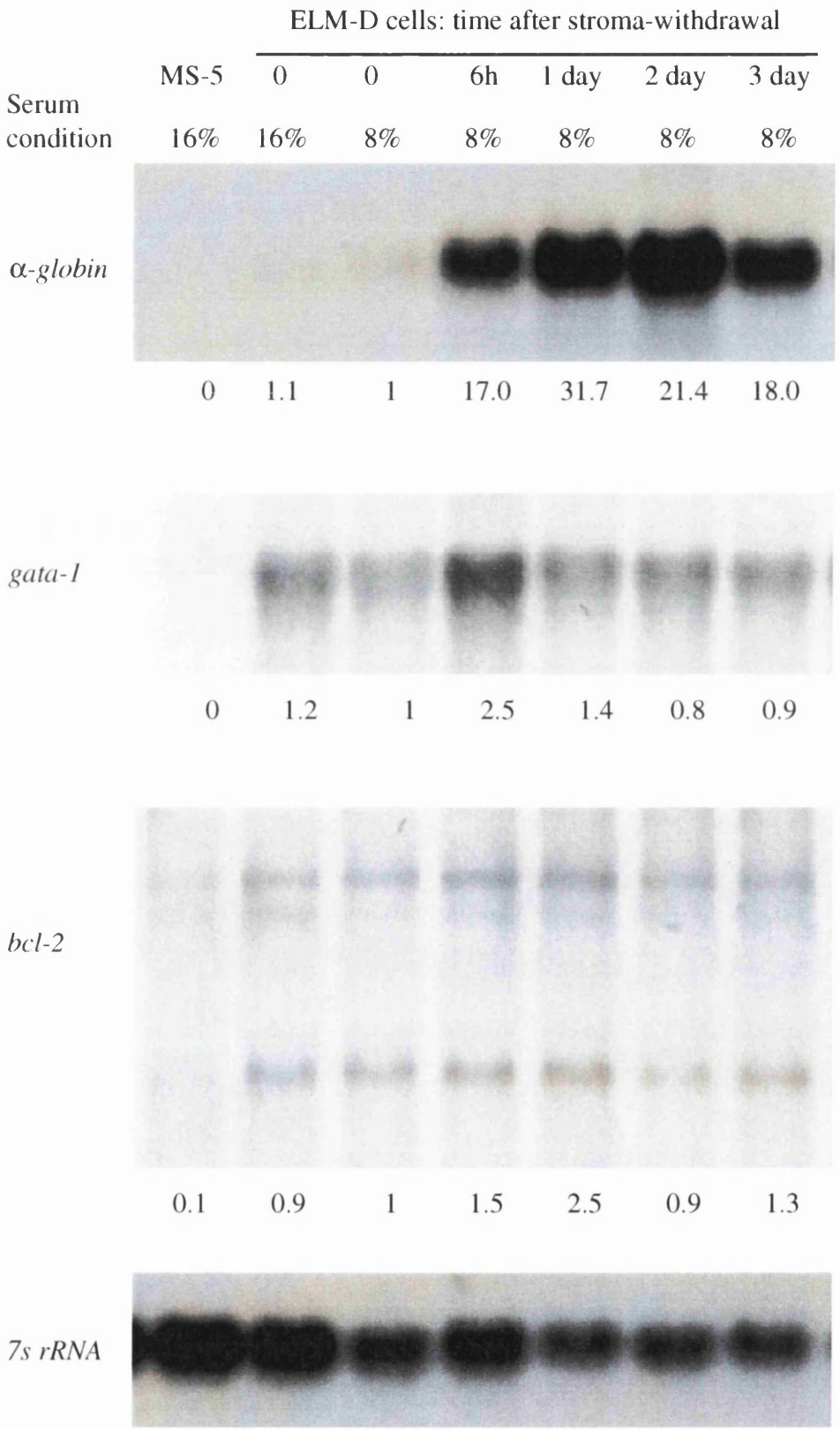
9.5.2. Changes in the *α-globin* and *bcl-2* expression of ELM-D cells upon stroma-withdrawal in 8% serum.

Figure 9.7 shows the data of a similar stroma-withdrawal experiment but the serum condition used was 8% rather than 16%. It can be seen that ELM-D cells also differentiate upon stroma-withdrawal in 8% serum, but to a much larger degree. At day 1 after stroma-withdrawal, the expression of *α-globin* increased to approximately 32 fold as that when the stroma was just removed. The fact that the increase in *α-globin* expression of ELM-D cells occurred more rapidly and to a larger extent in 8% serum may be due to the effects of serum on various sub-populations of the cells. First of all, the majority of the population, which is truly stroma-dependent, had less proliferation capability in lower serum and therefore their expansion was limited and differentiation enhanced. In the meanwhile, the proliferation of the stroma-independent sub-population of ELM-D cells was also largely reduced.

Changes in the *gata-1* mRNA expression was also measured. *Gata-1* is a transcription factor that positively regulates erythroid differentiation (see Section 2.2.1). It has been reported that the *gata-1* expression is up-regulated at an early stage in erythroid differentiation (Bockamp *et al.*, 1994). It can be seen that the expression of *gata-1* mRNA was transiently up-regulated as early as 6h after stroma-withdrawal.

On the other hand, although the *bcl-2* expression displayed a slight increase reaching about 2.6 fold as the original level at day 1, it decresed thereafter and remained at more or less the normal level. It should be noted that the maximum level of *α-globin* expression in ELM-D cells upon stroma-withdrawal was 31 fold in 8% serum, in contrast to 9 fold in 16% serum. On the other hand, the maximum level of *bcl-2* expression in ELM-D cells upon stroma-withdrawal was 2.6 fold in 8% serum, but 4.6-fold in 16% serum. If *Bcl-2* indeed plays a positive role in erythroid expression it would be expected that, upon stroma-withdrawal, ELM-D cells grown in 8% serum, which differentiated 3.5 times better than that in 16% serum, would display more increase in the *bcl-2* expression. This is in contrary to what was observed. Therefore, it seems unlikely that *Bcl-2* plays a positive role in erythroid differentiation. Rather, the increase

Figure 9.7. Changes in the α-*globin*, *gata-1* and *bcl-2* mRNA expression in ELM-D cells after stroma-withdrawal in 8% serum by Northern blotting analysis. The figures below the lanes are the relative levels of gene expression after calibration with the *7s rRNA* loading control.



in *bcl-2* expression in ELM-D cells upon stroma-withdrawal may indeed be a response to the deprivation of survival and proliferation signals. Factors in the serum may augment this response in favour of survival and growth of the cells.

In conclusion, ELM-D cells undergo terminal erythroid differentiation upon stroma-withdrawal. The *bcl-2* expression is only slightly up-regulated. which seems to be a result of the deprivation of the survival and proliferation signals that are normally provided by the stromal cells.

9.6. Conclusions.

Mass culture experiments have demonstrated that ELM-D cells are stroma-dependent. Upon stroma-withdrawal, the majority of the population dies out in the form characteristic of apoptosis. However, the fact that the *α-globin* expression in ELM-D cells is largely increased upon stroma-withdrawal points out that ELM-D cells undergo erythroid differentiation in the absence of stroma. The *bcl-2* expression in ELM-D cells only increases slightly at the early stage of stroma-withdrawal, and this may be a result of the deprivation of the survival and proliferation signals that are normally provided by the stroma.

In addition, the kinetics of cell death and differentiation, and the changes in gene expression in the stroma-withdrawal experiments are largely affected by the level of serum used. The reduced level of serum (8% instead of 16%) does not affect the ability of stromal cells to maintain the long-term growth of ELM-D cells but largely facilitates cellular and molecular studies.

CHAPTER 10: Gene Expression Studies of the ELM system

As mentioned in Chapter 6, various ELM cell lines are different in stroma-dependence, differentiation potential and tumorigenicity. This provides us a unique opportunity to identify genetic events involved in generating these different phenotypes. The genes that have been studied can be divided into four categories: (1) genes involved in the induction of erythroleukaemia (the *ets* family members, in particular, *erg* and *fli-1*, also see Chapter 4); (2) genes involved in the control of cell survival, especially when in the absence of survival and proliferation signals that are normally present (*bcl-2* and *bcl-x*, also see Chapter 5); (3) genes that play a crucial role in the regulation of erythroid differentiation (*gata-1* and *scl*, see Section 2.2); and (4) a gene that serves as a erythroid differentiation marker (*α-globin*).

For the gene expression studies, the stroma-dependent (ELM-D) and -independent cells (ELM-I/1, 2, 5 and 6) were used. They were seeded at a density of 10^5 per ml in the ELM growth medium containing 16% horse serum (see Methods and Materials) and harvested 3 days later, when they were still in logarithmic growth. Total mRNA and protein were isolated for Northern and Western blot analysis (see Methods and Materials). The ELM-D cells were grown on the MS-5 stromal cells, and at the time of harvest they were shaken off from the stroma. In this way, it is able to recover >90% of ELM-D cells with few stromal cell contamination. Even though, MS-5 cells were also included in gene expression studies.

10.1. Gene expression studies at the mRNA level.

In Northern blotting analysis, 20μg of total RNA was used and the blot was probed with ^{32}P-radiolabelled DNA fragments from the genes of interest (see Methods and Materials). RNA loading was assessed by stripping the blot of the radiolabelled probes and reprobing it with a 7s *rRNA* genomic fragment. Autoradiographs were scanned and quantitated by laser densitometer (see Methods and Materials). Relative levels of gene expression were calculated after calibration with the loading control. Representative blots were shown in Figure 10.1 and Table 10.1 summaries the relative levels of gene expression in the ELM system.

It can be seen from Figure 10.1 and Table 10.1 that, as reported by Dr. Nibbs (Nibbs *et al.*, 1993), that the stroma-independent variants (ELM-I/1, 2, 5 and 6) express an elevated level of *erg* in comparison to their stroma-dependent parent (ELM-D). More significantly, the expression of *fli-1* in ELM-I/1 cells is highly activated while the other ELM cells do not express *fli-1* at all.

Figure 10.1. Gene expression studies of the ELM system at the mRNA level by Northern blotting analysis: (a) *erg*, (b) *fli-1*, (c) *bcl-2*, (d) *bcl-x*, (e) *gata-1* and *scl/tal-1*, and (f) α-*globin*.

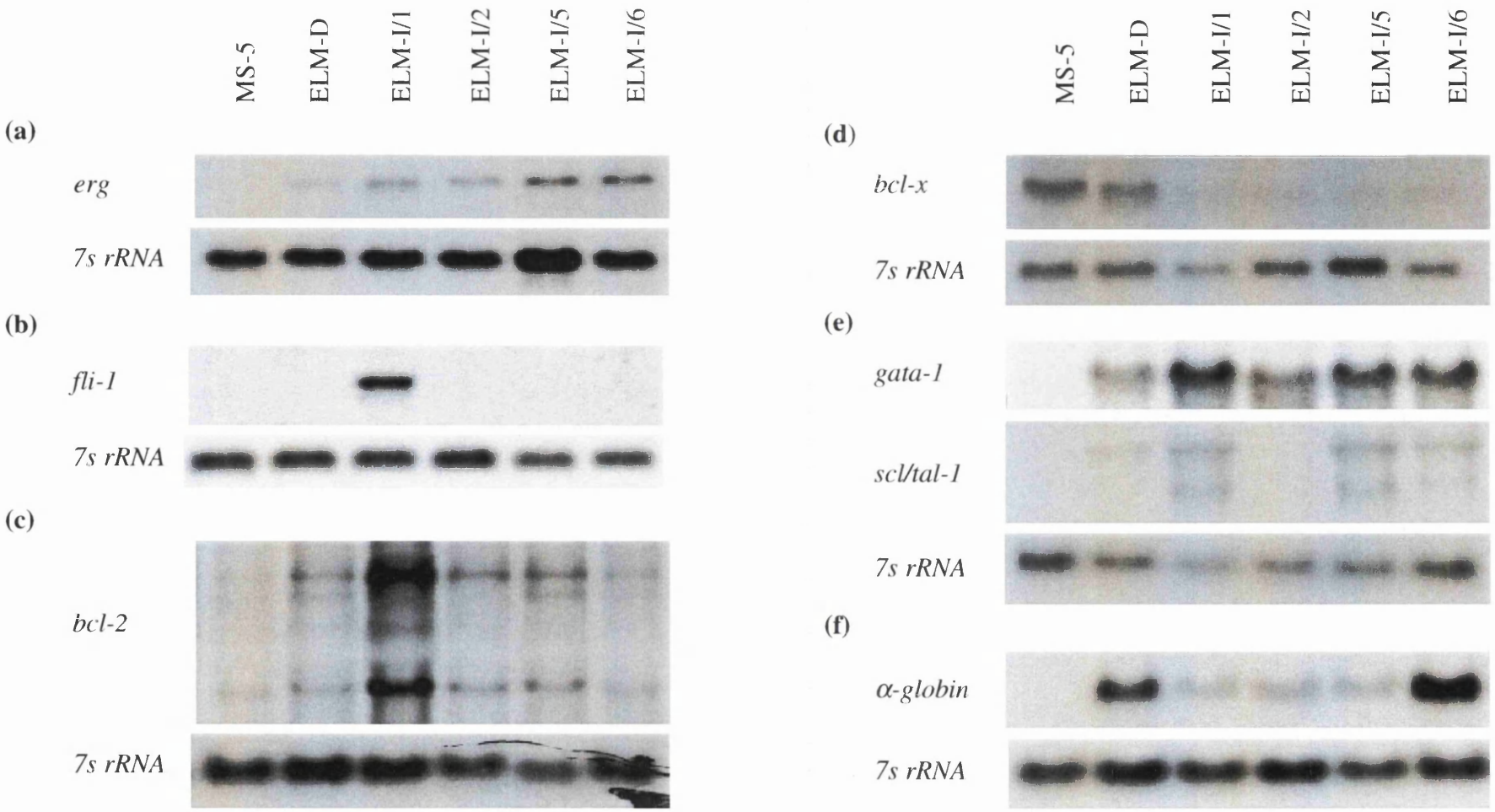


Table 10.1. Relative levels of gene expression in the ELM system. The figures given have been calibrated with the 7s *rRNA* loading control. The means and standard deviation are results of at least three independent experiments.

	MS-5	ELM-D	ELM-I/1	ELM-I/2	ELM-I/5	ELM-I/6
erg	0	1	6.0	5.5	5.4	7.3
fli-1	0	0	+++	0	0	0
bcl-2	0.5±0.1	1	11.3±4.6	2.6±0.8	3.1±2.7	3.1±1.0
bcl-x	1.7	1	0.3	0.4	0.4	0.4
gata-1	0	1	4.5±0.9	1.9±0.4	4.8±2.4	3.8±2.2
scl	0	1	8.4±3.5	0.4±0.3	7.8±2.1	1.5±0.7
α-globin	0	1	0.2±0.1	0.3±0.2	0.5±0.3	2.6±2.0

The second most significant feature of gene expression in the ELM system is that the *bcl-2* expression in ELM-I/1 cells is also highly up-regulated. It is approximately 11 fold as that in ELM-D cells, and about 4-5 fold as that in the other ELM-I cells. The expression of *bcl-x* in ELM-D is approximately 2.5-3 fold as that in ELM-I cells.

Differences in the *gata-1* and *scl* expression also exist among different ELM cell lines but the significance of them is difficult to assess. It is intriguing that ELM-I/1 cells, which are blocked in differentiation, express the highest levels of *gata-1* and *scl* mRNA. It would be interesting to see if this correlates with the expression at protein level, and if there is any mutations in these genes. It is possible that differentiation arrest in ELM-I/1 cells results from either a lack of transcription factors that positively regulate erythroid differentiation, such as *gata-1* and *scl*, or aberrant functional activities of those factors (discussed in detail in Chapter 13).

The expression of *α-globin* in ELM-I/1, I/2 and I/5 cells is only 1/5th to one-half of that in ELM-D cells, indicating that they are in a less differentiated state. The ELM-I/6 cells express higher levels of *α-globin* than that in ELM-D cells.

Characterisation of gene expression at the mRNA level had allowed us to postulate theories with regard to the molecular mechasisms of the stroma-dependent growth, differentiation arrest and tumourgenicity of the ELM system. It is possible that the elevated expression of *erg* in ELM-I cells is associated with the occurence of stroma-independent growth. Indeed, it has also been found that, upon stroma-withdrawal, the *erg* expression at the mRNA level in ELM-D cells was up-regulated (Nibbs *et al.*, 1993). Furthermore, activation of *fli-1* and up-regulation of *bcl-2* may be responsible for the differentiation arrest and increased tumorigenicity in ELM-I/1 cells. The fact that there are many potential *Ets* binding sites in a very important regulatory element (i.e.

NRE) within the *bcl-2* promoter (see Chapter 5.3) further points to a link between the activation of *fli-1* and *bcl-2*. To further pursue these possibilities, functional studies are desired and it is necessary that comparative studies of gene expression in the ELM system was extended to the protein level.

10.2. Gene expression studies at the protein level.

To determine whether differences in gene expression at the mRNA level observed in the ELM system correlate with the expression of proteins, the levels of *Erg*, *Fli-1* and *Bcl-2* proteins were analysed by Western blotting. Cells were cultured and harvested and total protein isolated as described earlier on in the chapter (also see Methods and Materials). 20μl of total protein extracts (10^7 cells / 100μl) were used for detection of the proteins in Western blotting analysis (see Methods and Materials), and the results are shown in Figure 10.2 and 10.3. It can be seen that ELMI/1 cells also express high levels of *Fli-1* and *Bcl-2* proteins. The *Fli-1* and *Bcl-2* proteins in other ELM cells are not readily detectable.

The expression of *Erg* proteins was detected by using a polyclonal rat-anti-human *Erg* antibody purchased from Santa Cruz, which recognises the human *Erg*, but not other *Ets* proteins. Its ability to cross-react with the murine *Erg* had not been tested. In order to test the cross-reactivity of the antibody, protein extracts from the human cells lines with known *Erg* expression profiles were included as controls. The KG-1 and Molt-4 cells, which express the *Erg* proteins (Prasad *et al.*, 1994), were used as the positive controls, whereas HL-60 and K562 cells, which do not express the *Erg* proteins, were used as the negative controls (Rao *et al.*, 1987; Duterque-Coquillaud *et al.*, 1993). Figure 10.3 shows the *Erg* protein expression in the ELM system. The *Erg* protein can be expressed in three different isoforms with molecular weights of 41 kD, 52 kD (normally occurs as a doublet) and 59 kD (Reddy and Rao 1991; Murakami et al., 1993; Siddique *et al.*, 1993), respectively. It can be seen from Figure 10.3(a) that there were two bands (or a doublet) around 52 kD in all the ELM cells, MS-5 cells, the two positive controls (KG-1 and Molt-4) and one of the negative controls (K562). Only the bottom band of the doublet was present in the other of the negative control (HL60). In addition, there are two bands around 41 kD in the two positive controls and, furthermore, the bottom one migrated at a similar position as the bottom band in ELM-I/1 cells. These two bands were not present in any of the other cell lines. The results with regard to the Erg expression in the positive control cell lines are consistent with what have been reported (Siddique *et al.*, 1993). However, the fact that the doublet around 52 kD was present in virtually all the cell lines including the negative controls raises the doubt with regard to

Figure 10.2. Expression studies of the *Fli-1* (a) and *Bcl-2* (b) proteins in the ELM system

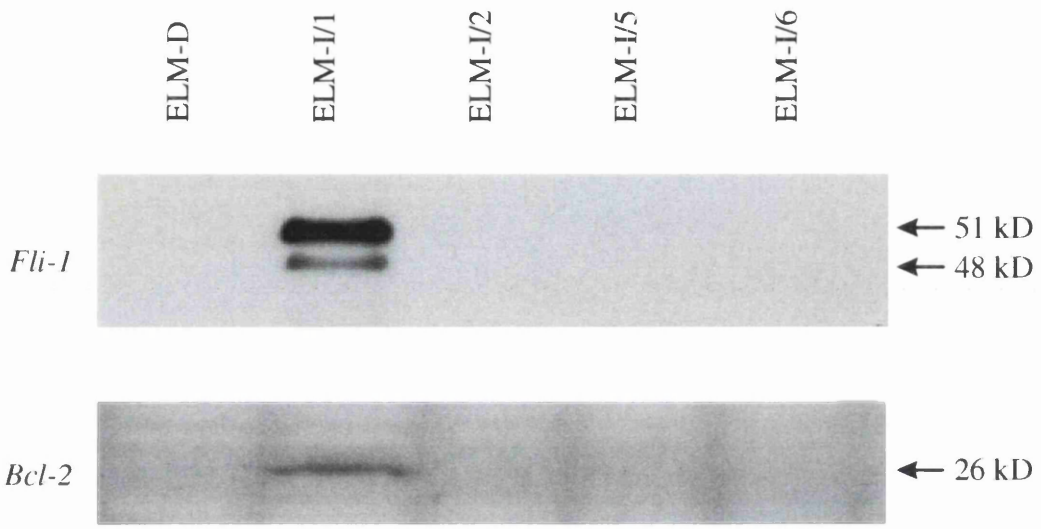
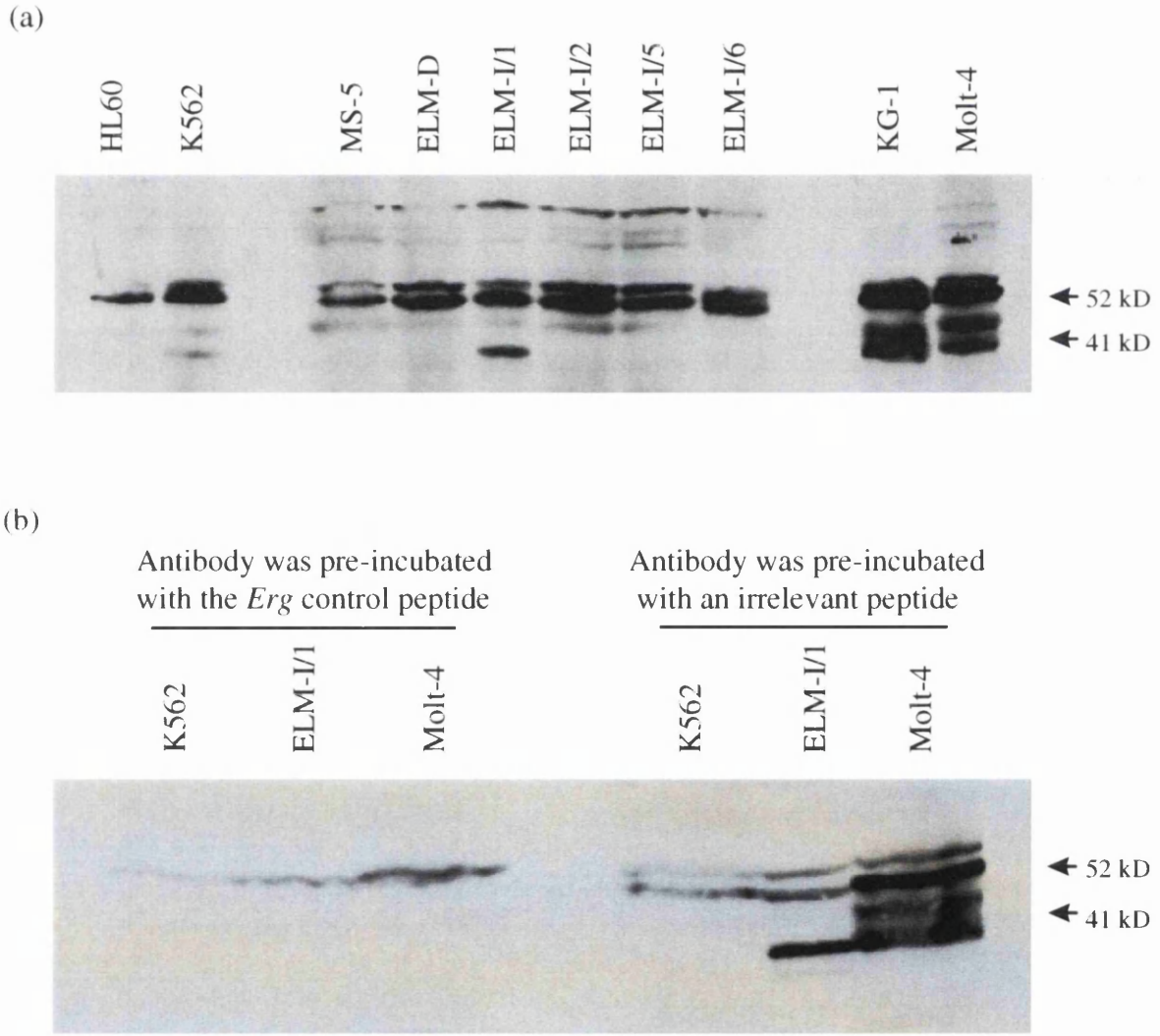


Figure 10.3. Expression of the *Erg* proteins in the ELM system.

(a) Western blotting analysis of total protein extracts from the ELM cells;
(b) Immuno-depletion and Western blotting analysis of the *Erg* expression in the ELM cells. The *Erg* protein was detected in Western blotting using the antibody that had been pre-incubated with the *Erg* control peptide (left panel) or an irrelevant peptide (right panel).

The ELM cell lines: ELM-D, ELM-I/1, I/2, I/5 and I/6
The stromal cell line: MS-5
Positive control cell lines: KG-1 and Molt-4
Negative control cell lines: HL60 and K562



whether or not these bands represented the *Erg* expression or, rather, they were non-specific.

The specificity of the antibody was further characterised by immuno-depleting the antibody with the human *Erg* control peptide (Santa Cruz) which was used as the antigen to generate the antibody. Immuno-depletion was carried out by incubating the antibody with 50μg of the control peptide on ice for 30min prior to application in the Western blotting analysis. As a control, an aliquot of the antibody was incubated with the same amount of an irrelevant peptide (*bcl-2* peptide) which was synthesised and purified in the same way by the same company. Figure 10.3(b) shows the results of such immuo-depletion and Western blotting analysis. The right panel shows that pre-incubating the antibody with irrelevant peptides gave the same banding pattern as that in Figure 10.3(a). The left panel shows that after immuno-depleting the antibody with the *Erg* control peptide, the top band of the doublet around 52 kD persisted, indicating that it was non-specific, whereas all the other bands disappeared. Although the result further confirms that the bands around 41 kD are likely to be specific, it still does not provide convincing evidence with regard to the specificity of the bottom band of the doublet around 52 kD. This is because that not only this band was present in the negative control (Figure 10.3b, right panel) but also it disappeared when the antibody was immuno-depleted with the *Erg* control peptide (Figure 10.3b, left panel). It is more likely that it is non-specific. Therefore, in conclusion, none of the ELM cells apart from ELM-I/1 express detectable levels of the *Erg* protein. In ELM-I/1 cells, an *Erg* protein of approximately 41 kD is expressed.

10.3. Conclusions.

The most significant results from the gene expression studies of the ELM system can be summarised as the following: (1) The stroma-independent cells (ELM-I/1, 2, 5 and 6) express an elevated level of the *erg* mRNA in comparison to their stroma-dependent parent (ELM-D). However, only the *Erg* protein in ELM-I/1 cells is readily detectable; (2) The expression of *fli-1* at both mRNA and protein levels in ELM-I/1 cells is significantly activated but is absent in all the other ELM cells; (3) The *bcl-2* expression at the mRNA level in the stroma-independent cells is higher than that in ELM-D cells, especially in ELM-I/1 cell line which is the only cell line wherein the *Bcl-2* protein is expressed at a detectable level.

These conclusions have led to three lines of potential functional work. The first aimed at clarifying the possible roles of *Erg* in confering stroma-dependent growth. The second was to test the possible involvement of *Bcl-2* and *Fli-1* in differentiation arrest and increased tumorigenicity in ELM-I/1 cells. The functional studies involving *Fli-1*

have been carried out by Dr. Bartholomew in the laboratory. These could be carried out either by over-expression or antisense approaches. The third was to test whether or not *Fli-1* can transactivate the *bcl-2* promoter and is responsible for the up-regulation of *bcl-2* in ELM-I/1 cells.

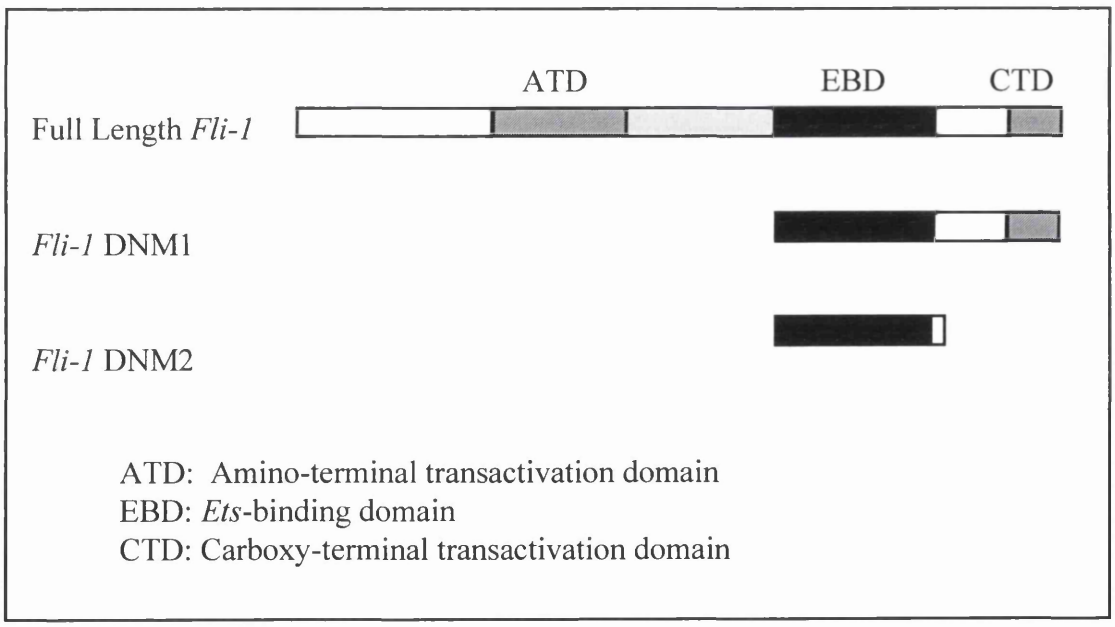
CHAPTER 11: Molecular Mechanisms of *bcl-2* Up-regulation in ELM-I/1 Cells.

11.1. Transactivation of the *bcl-2* promoter by *Fli-1*.

11.1.1. Transactivation of the *bcl-2* promoter by *Fli-1* in a quail fibroblast assay system.

It has been established that in ELM-I/1 cells, *fli-1* and *bcl-2* are significantly up-regulated at both mRNA and protein levels (see Chapter 10). This, together with the fact that an important regulatory element (NRE) in the *bcl-2* promoter contains multiple potential Ets-binding sites (EBS) (see Figure 5.1), suggested that it might be worthwhile to determine whether *Fli-1* can transactivate the *bcl-2* promoter, and whether *Fli-1* is responsible for the up-regulation of *bcl-2* expression in this particular cell line. A murine *fli-1* expression vector, pRC-CMV-*fli-1*, was used in transient transfection assays, where expression of the *fli-1* cDNA is driven by the cytomegalovirus promoter. Two truncated *Fli-1* mutants, DNM1 and DNM2, were also used (Figure 11.1): DNM1 includes the last 175 amino acids from the carboxyl-terminal, which contains the DNA-binding domain and the minor carboxyl-transactivation domain; while DNM2 contains the DNA-binding domain only. It was expected that DNM1 retains part of the transactivation activity compared with the full length *Fli-1* protein, whereas DNM2 should not have any transactivation activity at all.

Figure 11.1 Schematic representation of the functional domains of the murine *Fli-1* protein and its two truncated mutants.



To check that the assay system was functional correctly, the transactivation activity of the full length *Fli-1* protein and its truncated mutants thus generated was first tested using reporter vectors containing the luciferase cDNA under the control of the minimum herpes thymidine kinase (tk) promoter with or without insertions of three copies of the E74 target sequence upstream of the tk promoter. The E74 oligonucleotide contains the consensus sequence required for binding to the Drosophila *E74* protein (Urness and Thummel, 1990). A number of *Ets* proteins, including the human *Erg-1*, *Erg-2*, *Ets-1*, *Ets-2*, *Fli-1* and *Spi-1/PU.1*, can bind to and transactivate through this sequence (Reddy and Rao, 1991; Chang *et al.*, 1993; Rao *et al.*, 1993). A mutant E74 sequence (mE74) has also been derived where the core consensus sequence GGAA is mutated to CAA, which abolishes its binding activity to the *Ets* proteins (Nibbs *et al.*, 1993). Thus, the E74 sequence provides a very useful tool for assaying the transactivation activity of *Fli-1*. The *fli-1* expression vector and the E74 reporter construct were co-transfected into a quail fibroblast cell line, Q2BN. Q2BN has been chosen because it is highly transfectable, and Dr. Frampton has successfully applied this system in his studies on a variety of both chicken and murine *Ets* proteins. On the other hand, transfection efficiency of the ELM system was too low to conduct such assays.

In order to be certain that any changes in the reporter gene expression was due to the specific effect of co-transfection of the expression vector of *fli-1* or its truncated mutants, rather than due to differences in transfection efficiency or any other artefacts, a β-galactosidase expression vector, pRSV(β-*gal*), was co-transfected to serve as an internal control, in which the β-*gal* cDNA is driven by the promoter of Rous Sarcoma Virus. It had been confirmed that co-expressing the *fli-1* expression vector, over the range applied in our assays, i.e. 0-1μg, does not have considerable effect on the RSV promoter (Figure 11.2a). However, it does have significant effects on the HSV and GK promoters (Figure 11.2b and 11.2c). Based on this analysis, 1μg of the pRSV(β-*gal*) vector was co-transfected to serve as an internal control in all the assays, unless otherwise indicated.

Figure 11.3a shows the luciferase expression from various reporter constructs when co-expressed with either the expression vectors (pRC-CMV(*fli-1*) or pRC-CMV) or a non-expression vector (the Bluescript vector, Promega). The use of the Bluescript vector served as an additional control to test whether the expression vectors used had brought about certain artefacts. It shows that expression of the *Fli-1* protein led to an average of 2-fold increase in the luciferase expression (Figure 11.3a) from the reporter construct pGL2(min.tk+3xE74) containing the minimum tk promoter linked to three copies of E74 sequences, compared with the negative controls where either the empty expression vector (pRC-CMV) or a non-expression vector (Bluescript) was used (Figure 11.3a).

Figure 11.2. Effects of *Fli-1* on the expression of a β-galactosidase (β-*gal)* reporter gene driven by (a) RSV, (b) HSV, and (c) GK promoters.

To test the effect of *Fli-1* on the expression of β-*gal* by various expression constructs and, therefore, the validity of choosing a particular construct in transient transfection studies as the internal control, 1μg of β-*gal* expression vectors (pRCV(β-*gal*), pHSV(β-*gal*) and pGK(β-*gal*)) and various amounts (0-5μg) of the *fli-1*expression vector (pRC-CMV-*fli-1*) were co-transfected into Q2BN cells by the traditional calcium-phosphate method (see Methods and Materials). Carrier DNA, the pRC-CMV vector, was used so that the same amount of DNA was used in each transfection. Cells were harvested after 48h and the β-*gal* assays performed (see Methods and Materials). Cell lysates prepared from pRSV(β-*gal*) transfectants were incubated with the substrates of β-*gal* for 25 min before reactions were stopped and OD(420nm) was read, whereas in the case of pHSV(β-*gal*) and pGK(β-*gal*), an incubation time of 90 min was used since the β-*gal* expression driven by the HSV and GK promoters was less efficient. The results represent the means and standard deviation of two independent experiments, each performed in triplicates.

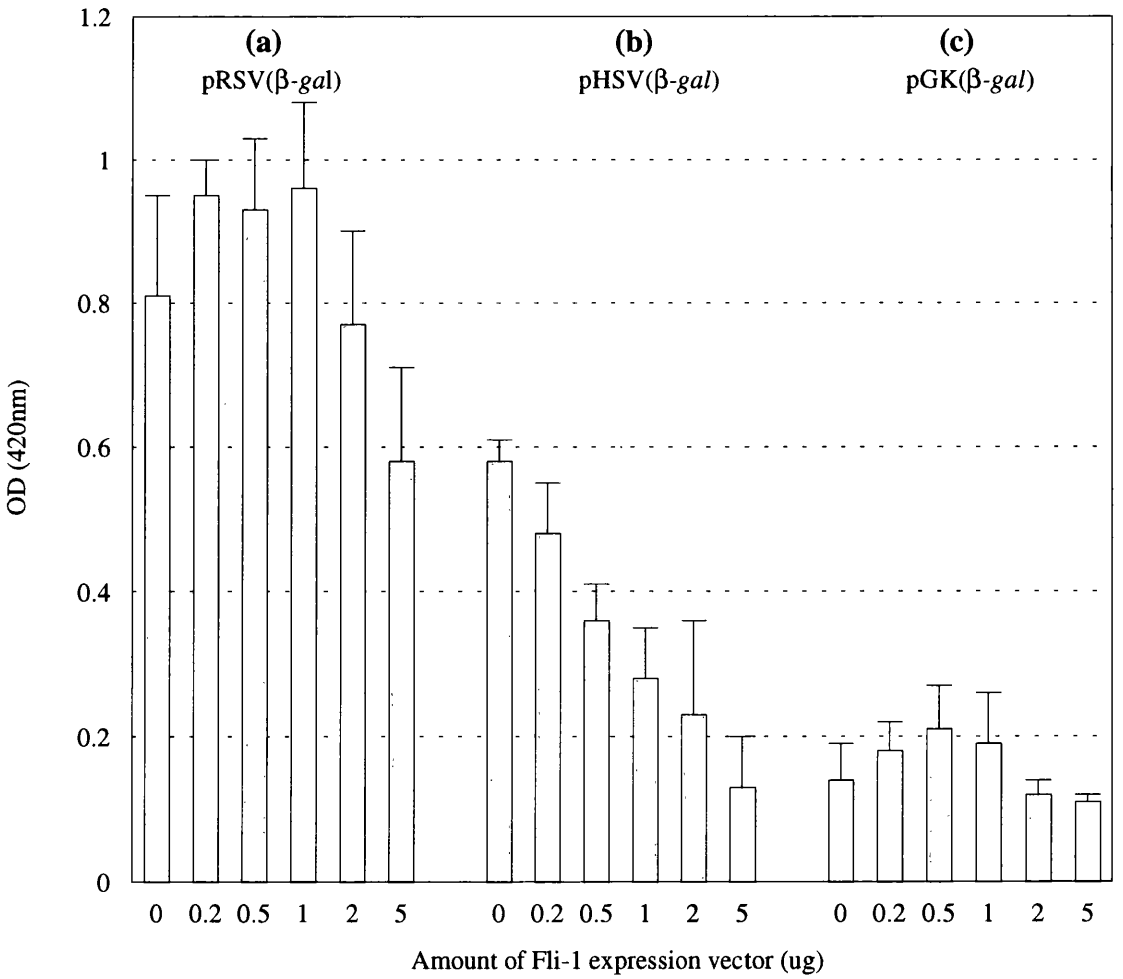


Figure 11.3. Effects of *Fli-1* and its truncated mutants, DNM1 and DNM2, on the activity of the E74-linked minimum thymidine kinase (tk) promoter.

The wild-type *Fli-1* protein (a) but not its truncated mutants (b) can transactivate the minimum tk promoter via the linked three copies of E74 sequences. (c) When co-expressed with wild-type *Fli-1* protein, the *Fli-1* truncated mutant, DNM2, which only contains the DNA-binding domain, can act as a dominant negative mutant and reduce the transactivation of the E74-linked minimum tk promoter by *Fli-1*. 1μg of pRSV(β-gal) was co-transfected as the internal control for transfection efficiency. The luciferase activity was then divided by the β-galactosidase activity to normalise transfection efficiency. The results represent the means and standard deviation of two independent experiments, each performed in triplicates.

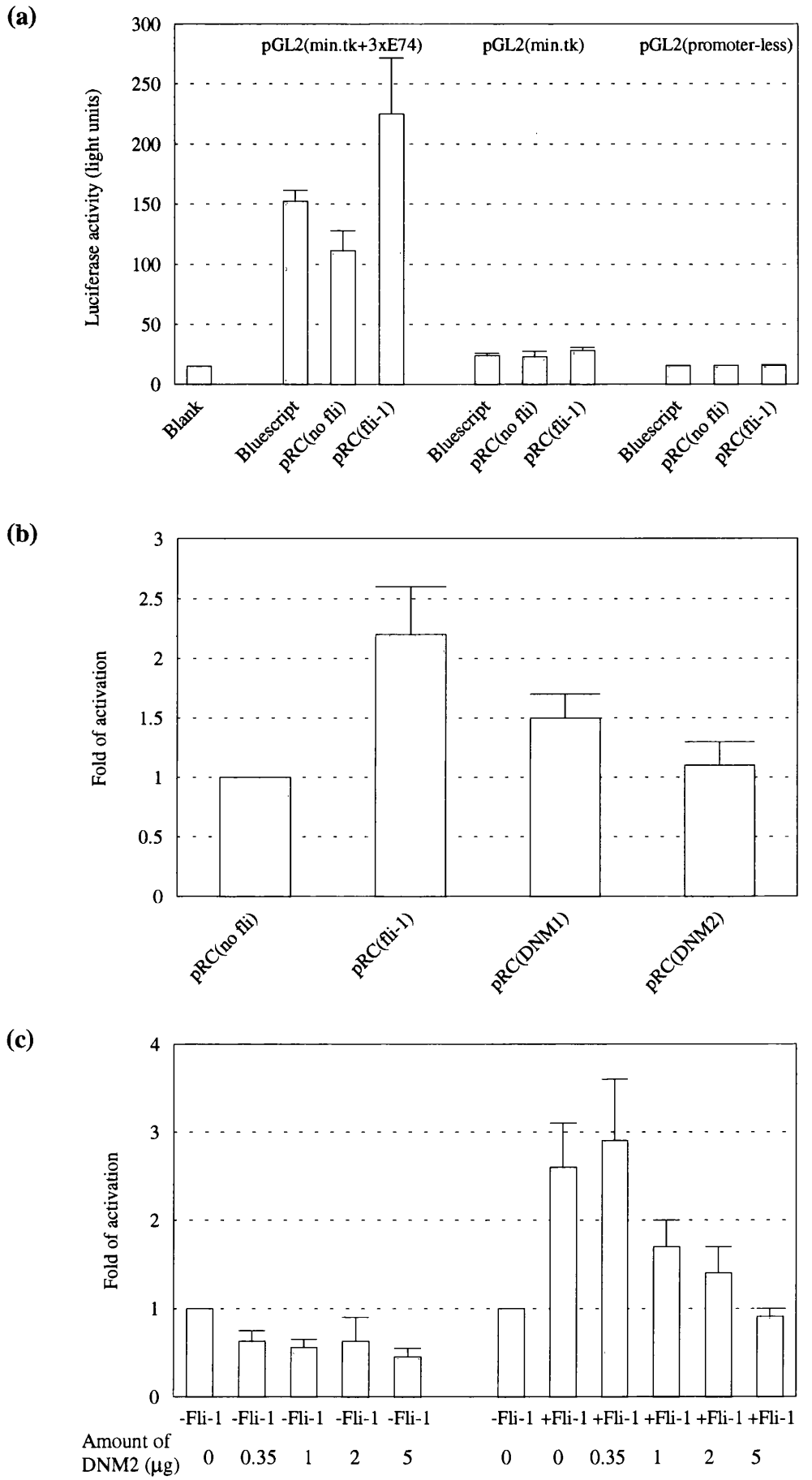
(a) 250ng of reporter constructs (pGL2(3xE74+min.TK), pGL2(min.tk) or pGL2(promoter-less)) and 150ng of the *fli-1* expression vector (pRC-CMV-*fli-1*) were co-transfected into Q2BN cells by the traditional calcium-phosphate method (see Methods and Materials). The negative controls include using the empty expression vector (pRC-CMV, indicated as "pRC (no *fli-1*)") or a non-expression vector plasmid (the Bluescript vector). Cells were harvested 48h post-transfection and luciferase assays performed (see Methods and Materials).

(b)150ng of the E74-linked minimum tk promoter-reporter construct (pGL2(3xE74+ min.tk)) and 350ng of the pRC-CMV expression vectors, containing either *fli-1*, DNM1 or DNM2, were co-transfected into Q2BN cells. The empty expression vecotr, pRC-CMV (indicated as "pRC (no *fli-1*)") was included as the negative control. Cells were harvested and assayed for luciferase activity as above.

(c) Left panel: 150ng of the E74-linked tk promoter-reporter construct (pGL2(3xE74+ min.tk)) and various amounts (0-5μg) of the DNM2 expression vector were co-transfected into Q2BN cells, and cells were harvested and assayed for luciferase activity as above.

Right panel: 150ng of the E74-linked minimum tk promoter-reporter construct (pGL2(3xE74+ min.tk)), 350ng of the wild-type *fli-1* expression vector (pRC-CMV-*fli-1*) and various amounts (0-5μg) of the DNM2 expression vector (pRC-CMV-DNM2) were co-transfected into Q2BN cells, and cells were harvested and assayed for luciferase activity as above. Carrier DNA (pRC-CMV) was used so that the same amount of DNA was used in each transfection.

Figure 11.3


(a)
Luciferase activity (light units)
300
250
200
150
100
50
0
pGL2(min.tk+3xE74)
pGL2(min.tk)
pGL2(promoter-less)
Blank
Bluescript
pRC(no fli)
pRC(fli-1)
Bluescript
pRC(no fli)
pRC(fli-1)
Bluescript
pRC(no fli)
pRC(fli-1)
(b)
Fold of activation
3
2.5
2
1.5
1
0.5
0
pRC(no fli)
pRC(fli-1)
pRC(DNM1)
pRC(DNM2)
(c)
Fold of activation
4
3
2
1
0
-Fli-1 -Fli-1 -Fli-1 -Fli-1 -Fli-1
-Fli-1 +Fli-1 +Fli-1 +Fli-1 +Fli-1 +Fli-1
Amount of DNM2 (μg)
0 0.35 1 2 5
0 0 0.35 1 2 5

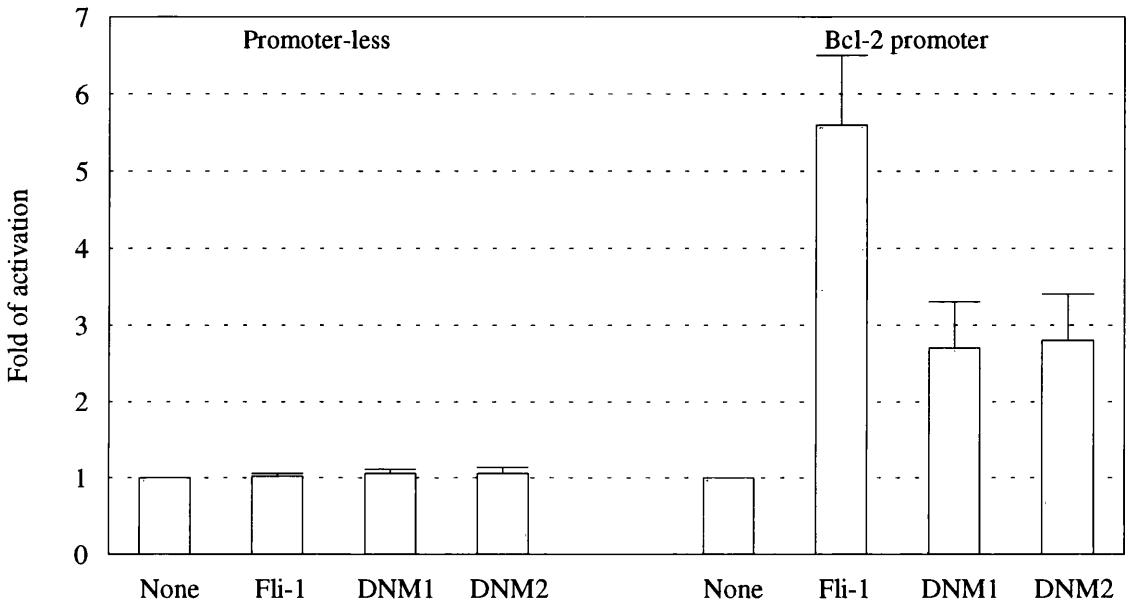
This increase was not observed with the reporter construct where the 3xE74 sequences are deleted (pGL(min.tk)), or with the promoter-less reporter where both 3xE74 and the minimum tk promoter were deleted (pGL2(promoter-less)) (Figure 11.3a). This indicates that the increase in the reporter gene expression observed is a specific effect due to the interaction between *Fli-1* and the 3xE74 sequence. Similar experiments using the *Fli-1* truncated mutants showed that DNM1, which retains the DNA-binding domain and the minor carboxyl-terminal transactivation domain, had reduced transactivation activity; while DNM2, which contains the DNA-binding domain only, did not have any transactivation activity at all (Figure 11.3b). Furthermore, DNM2 had, indeed, a dominant negative effect on the full length *Fli-1* protein. It can be seen from Figure 11.3c (right panel) that, with increasing amount of the DMN2 expression vector, the transactivation activity of full length *Fli-1* protein was reduced, and completely abolished when 5μg of the DNM2 vector was used. The control experiment presented in the left panel shows that, in the range used (i.e. 0-5μg), DNM2 on its own had no significant effect on the E74 reporter. Taken together, the various *Fli-1* expression vectors can produce the *Fli-1* proteins (i.e. the full length *Fli-1*, DNM1 and DNM2) with expected transactivation activity

In order to test whether *Fli-1* can transactivate the *bcl-2* promoter, we had tried to obtain the murine genomic *bcl-2* clone from the laboratory that originally cloned and characterised the gene but were fruitless. Attempts had also been made to isolate the full length *bcl-2* promoter from murine genomic DNA by the PCR approach but these proved unsuccessful, possibly due to the high GC content of the sequence. Instead, a partial *bcl-2* promoter containing 518bp upstream of the ATG start codon was isolated. Since this region of the *bcl-2* promoter displays the highest nucleotide sequence homology between mice and human in the non-coding regions, and it also contains the P2 promoter and a very important regulatory element implicated in transcriptional control of the gene (Young and Korsmeyer, 1993 and Miyashita *et al.*, 1994; also see Figure 5.1 and Figure 11.6), we decided to use this region as a starting point in the studies. The promoter-reporter construct (pXP2(m500)) was generated, which contains the luciferase cDNA under control of the partial *bcl-2* promoter, and the promoter-less reporter (pXP2) was used as the negative control. The left panel of Figure 11.4 indicates that the various *fli-1* expression vectors did not have any effect on the promoter-less reporter construct (pXP2). However, expression of the full length *Fli-1* protein led to an average of 5.5-fold increase in the *bcl-2* promoter activity in comparison to the empty expression vector (pRC-CMV) (Figure 11.4, right panel). Both the two truncated mutants, DNM1 and DNM2, had a reduced transactivation activity towards the *bcl-2* promoter (Figure 11.4, right panel). It is surprising that DNM2 had any transactivation activity at all since, as described earlier that, it only contains the DNA-binding domain. Such effects of the wild-type and mutant *Fli-1* proteins on the *bcl-2* promoter activity

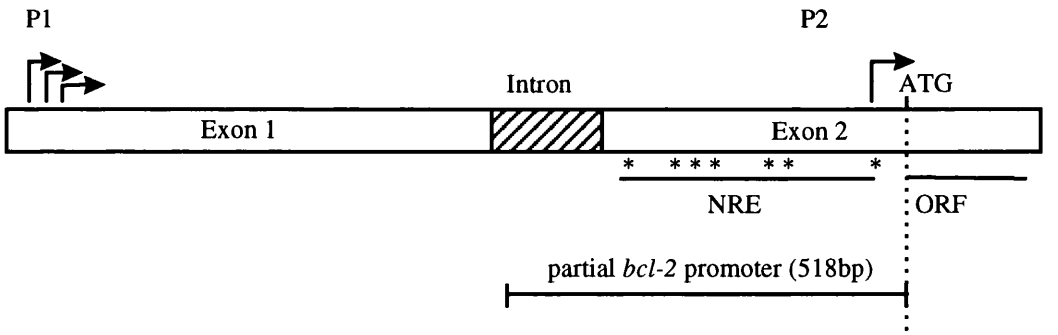
Figure 11.4. (a) Effects of *Fli-1* and its truncated mutants, DNM1 and DNM2, on the activity of a partial *bcl-2* promoter. (b) Schematic representation of the *bcl-2* promoter. The partial *bcl-2* promoter covers the 518bp region upstream of the ATG start codon.

150ng of the reporter constructs (either the promoter-less reporter, pXP2, or the *bcl-2* promoter-reporter, pXP2(m500)) and 350ng of either of the expression vectors were co-transfected into Q2BN cells by the traditional calcium-phosphate method (see Methods and Materials). The empty expression vector, pRC-CMV (as indicated by "None") was used in the negative controls. Cell were harvested 48h post-transfection and luciferase assays performed (see Methods and Materials). The results represent the means and standard deviation of three independent experiments, each performed in triplicate.

(a)



(b)



P1: Promoter 1
P2: Promoter 2
NRE: Minimum negative regulatory element
ORF: Open reading frame
ATG: Start codon
* : Putative *Ets*-binding site

can also be observed when in a range of expression vectors was used. Figure 11.5a shows the results of titration experiments using various amounts (i.e. 0-1μg) of *Fli-1*/DNM1/DNM2 expression vectors with a fixed amount of reporter construct. It can be seen that, with increasing amounts of wild-type *Fli-1* expression vector, the *bcl-2* promoter activity increased initially, peaked at around 200-500ng, and then decreased when more expression vector was used (Figure 11.5a, left panel). This bell-shaped curve of promoter activity in such expression vector titration experiments is typical, and presumably is due to the "squelching" effect. This occurs when over-expression of a transcription factor leads to its non-specific interaction with basal transcription factors, thereby sequestering them and thus reduce the promoter activity. Both DNM1 and DNM2, at various amounts used, had much reduced transactivation activity towards the *bcl-2* promoter (Figure 11.5a, middle and right panel). A different way of plotting Figure 11.5a gives clearer comparison of transactivation activity of the wild-type *Fli-1* protein and its truncated mutants at various amounts of expression vector used (Figure 11.5b).

In order to define the minimum promoter region required for *Fli-1* transactivation, deletion mutagenesis was desired to generate a shorter murine *bcl-2* promoter. As the 518bp region of the *bcl-2* promoter we had focused on in this study is 77.6% homologous to the human counterpart (Figure 11.6). Furthermore, the important regulatory element of the *bcl-2* promoter, which begins at 287bp upstream of the start codon, is 80.5% homologous between murine and human. Therefore, we took advantage of the fact that the promoter reporter constructs (pXP2(h300) and pXP2(h600)) were readily available, which contain the partial human *bcl-2* promoter 300bp and 646bp upstream of the start codon, and used them for comparison of their activity in response to the *Fli-1* expression. Figure 11.7 shows that *Fli-1* was able to transactivate the h300 and h600 promoters equally well. This indicates that the major elements required for the *Fli-1* transactivation of the partial human *bcl-2* promoter lies in the 300bp region upstream of the start codon, which conincides with the important regulatory element previously reported (Young *et al.*, 1993 and Miyashita *et al.*, 1994). The high homology of this region between the murine and human sequence also suggests the importance of this element in the transcriptional regulation of the gene, and that the minimum *bcl-2* promoter for murine may also lie in this region. Interestingly, there are seven potential *Ets*-binding sites in this region (Figure 5.1) and, consequently, we attempted to map the *Fli-1* activating sites within this region by DNase I footprinting analysis and gel retardation analysis.

Figure 11.5. Titration experiments of the effects of *Fli-1* and its truncated mutants, DNM1 and DNM2, on the *bcl-2* promoter activity.

150ng of the *bcl-2* promoter reporter constructs (pXP(m500)) and various amounts of (0-1μg) expression vectors (either pRC-CMV-*fli-1*, -DNM1, or DNM2) were co-transfected into Q2BN cells by the traditional calcium-phosphate method (see Methods and Materials). Carrier DNA (pRC-CMV) was used so that the same amount of DNA was used in each transfection. Cells were harvested 48h post-transfection and luciferase assays performed (see Methods and Materials). The results represent the means and standard deviation of four independent experiments, each performed in triplicates. It should be noted that (a) and (b) are based on the same data but plotted in different ways.

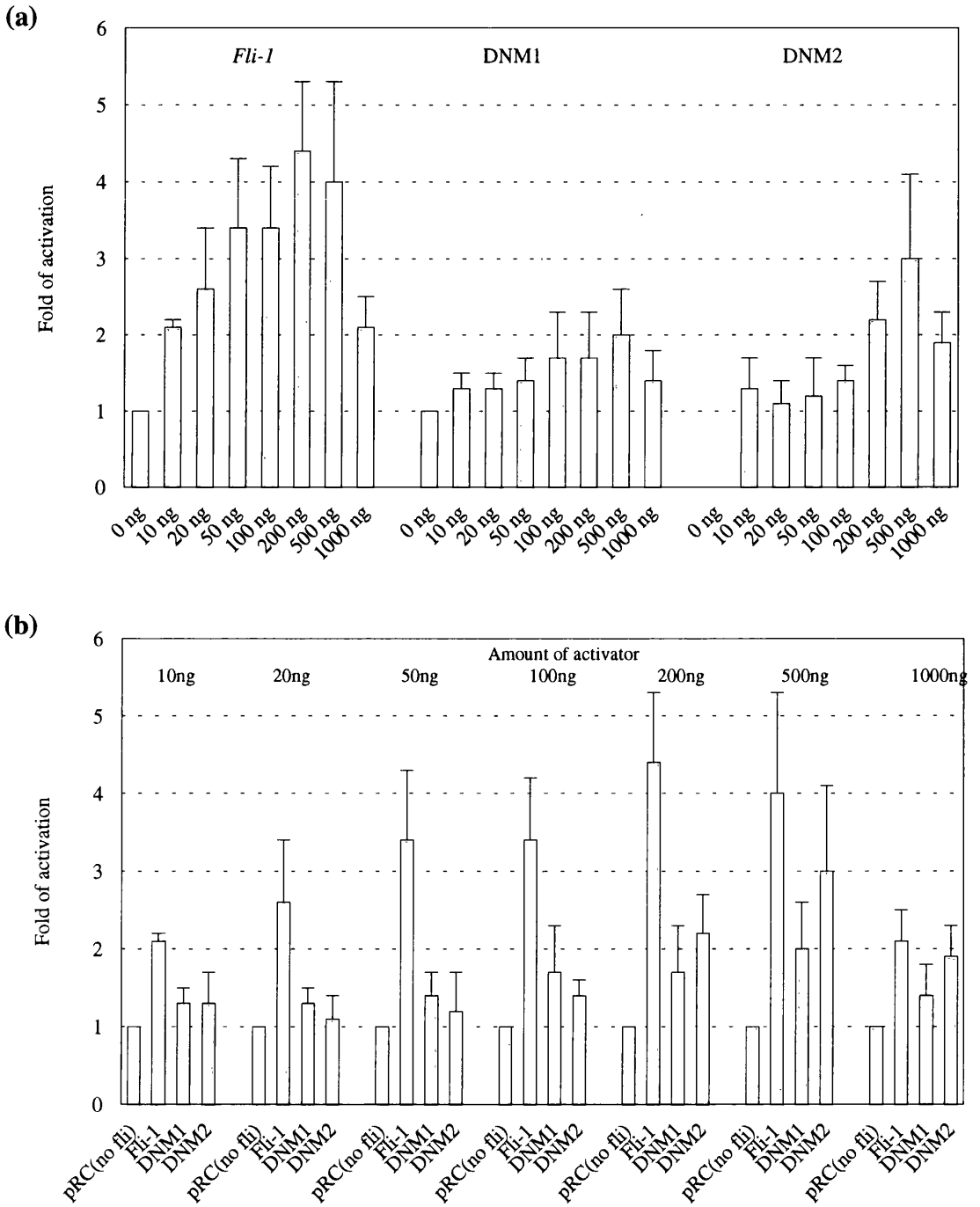


Figure 11.6 Sequence alignment between the murine and human *bcl-2* promoters studied. The region in bold indicates the regulatory element that is crucial for the transcriptional regulation of *bcl-2*. This region has been focused in subsequent DNase I footprinting and gel retardation analysis. The arrow indicates the transcriptional start site. The ATG start codons are boxed.

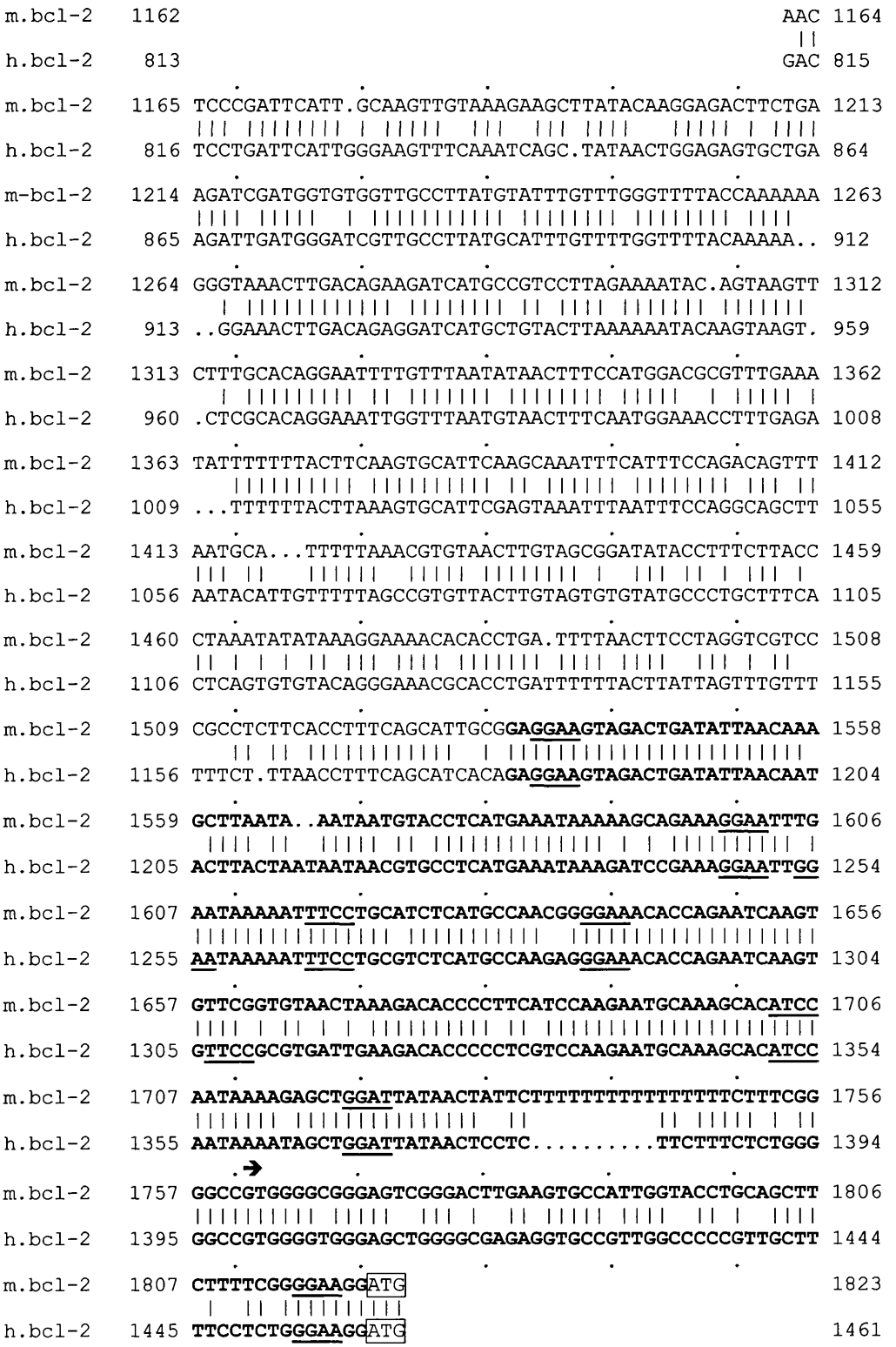
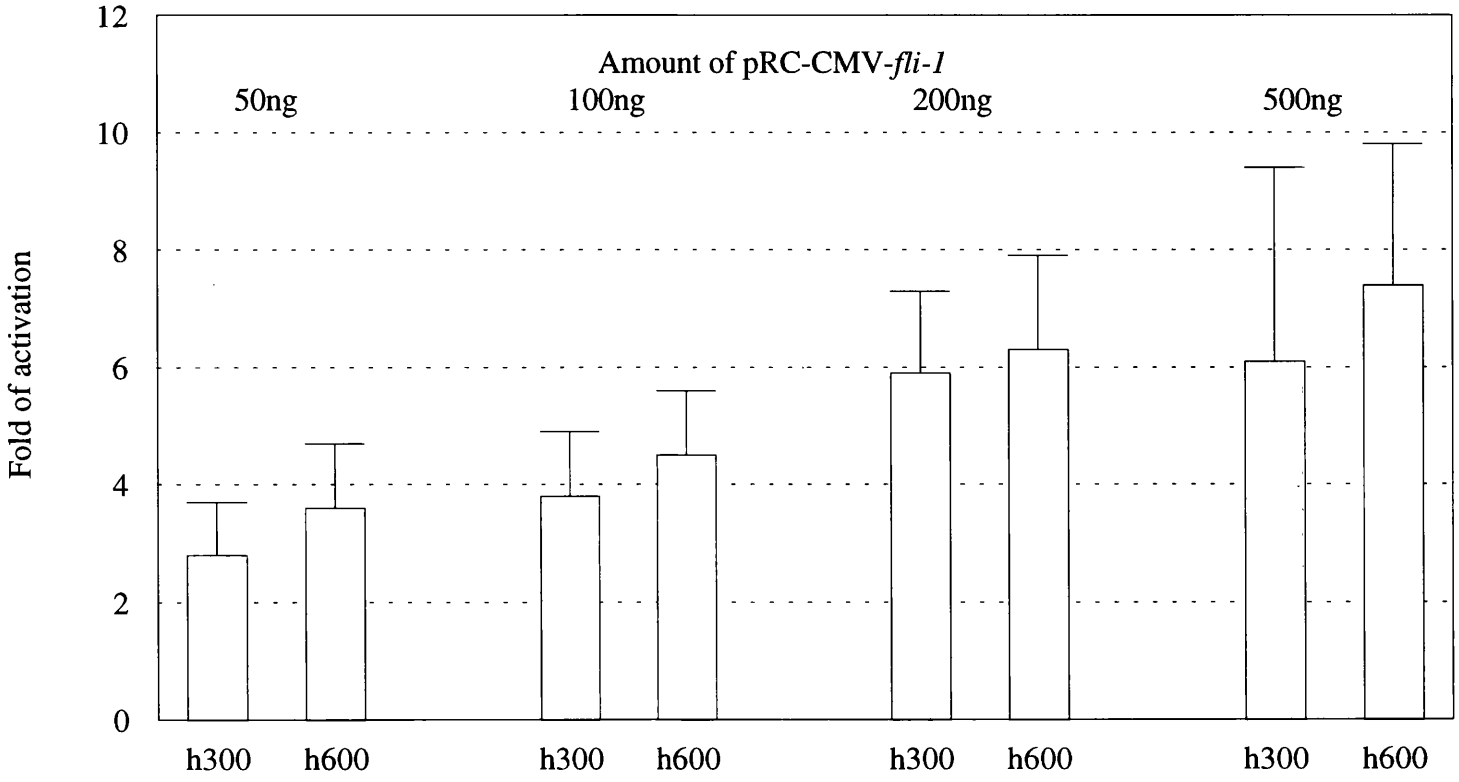


Figure 11.7. Comparison of the effects of *Fli-1* on the activity of two different human *bcl-2* promoters.

150ng of the promoter reporter construct (either pXP2(h300) or pXP2(h600)) and various amounts (0-500ng) of the *fli-1* expression vector (pRC-CMV-*fli1*) were co-transfected into Q2BN cells by the traditional calcium-phosphate method (see Methods and Materials). The pXP2(h300) and pXP2(h600) promoter reporters contain the luciferase cDNA under the control of the human *bcl-2* promoter region 300bp or 600bp upstream of the ATG start codon. Carrier DNA (pRC-CMV) was used so that the same amount of DNA was used in each transfection. Cells were harvested 48h post-transfection and luciferase assays performed (see Methods and Materials). The results represent the means and standard deviation of two independent experiments, each performed in triplicates.



11.1.2. The *Bcl-2* promoter activity in the ELM system.

The above evidence demonstrates that *Fli-1* can transactivate the *bcl-2* promoter in a quail fibroblast assay system. As the first step to prove that this is also the case in the ELM system, the *bcl-2* promoter activity was compared in ELM-I/1 cells that express the *Fli-1* protein and I/2 cells that do not. 25μg of the *bcl-2* promoter reporter construct, pXP2 (m500), was transfected into ELM-I/1 and I/2 cells by electroporation and cells were harvested after 48h for luciferase assay (see Methods and Materials). The transfection efficiency of ELM cells was poor but had been optimised to an acceptable degree to perform the assay. 25μg of the luciferase expression construct, pGL2 (min.tk), was used in parallel as a control for transfection efficiency, since the transfection efficiency was not high enough to enable the β-*gal* expression to be detectable using pRSV(β-*gal*) while luciferase assays are much more sensitive. It was demonstrated earlier (Figure 11.3a, middle panel) that *Fli-1* has no considerable effects on the minimum tk promoter.

Figure 11.8 shows that the tk promoter activity as measured by luciferase activity was similar in ELM-I/1 and I/2 cells. However, the *bcl-2* promoter acitivity in ELM-I/1 cells was approximately 4 times as that in I/2 cells. It is likely that transcription factor(s) that are present in ELM-I/1 but not I/2 cells are responsible for this difference. In light of the fact that *Fli-1* can transactivate the *bcl-2* promoter in the quail system, the *Fli-1* protein is a very likely candidate.

11.2. Preparation and characterisation of the nuclear extracts from ELM-I/1 and I/2 cells: quantitation and expression of the *Fli-1* protein.

Nuclear extracts were prepared from ELM-I/1 cells, which express the *Fli-1* protein, and ELM-I/2 cells, that do not. As DNA-binding activity of the ELM-I/1 and I/2 nuclear extracts will be compared in both footprinting and gel retardation analysis, quantitation of the extracts is important. This was carried by a combination of the BCA protein assays and gel retardation analysis using the CAAT oligonucleotide as the probe. The BCA assay is an accurate and reproducible method for quantitating total proteins. However, nuclear protein preparations unavoidably contain contaminations of cytoplasmic proteins, and the extent of contaminations can vary among different preparations. One way to compensate for this is to combine the BCA assays with gel retardation analysis using the CAAT oligonucleotide as the probe, which binds to ubiquitous transcription factor(s) and, therefore, can be used as the loading control of nuclear proteins (Plumb *et al.*, 1989). Figure 11.9a shows the CAAT-binding of nuclear proteins from various preparations. The amount of nuclear proteins loaded had been adjusted according to the calculated protein concentrations as a result of BCA assays,

Figure 11.8. Comparison of the *bcl-2* promoter activity in ELM-I/1 cells that express the *Fli-1* protein, and ELM-I/2 cells that do not.

25μg of the circular *bcl-2* promoter reporter construct, pXP2(m500), was transfected into ELM cells by electroporation (see Methods and Materials). Since the transfection efficiency of ELM cells is too low to perform β-*gal* assays, pGL2(min.tk) was used in parallel as a control for transfection efficiency. pGL2(min.tk) contains the luciferase cDNA downstream of the minimum thymidine kinase (tk) promoter. Cells were harvested 48h post-transfection and luciferase assays performed (see Methods and Materials). The results represent the means and standard deviation of two independent experiments, each carried out in triplicates.

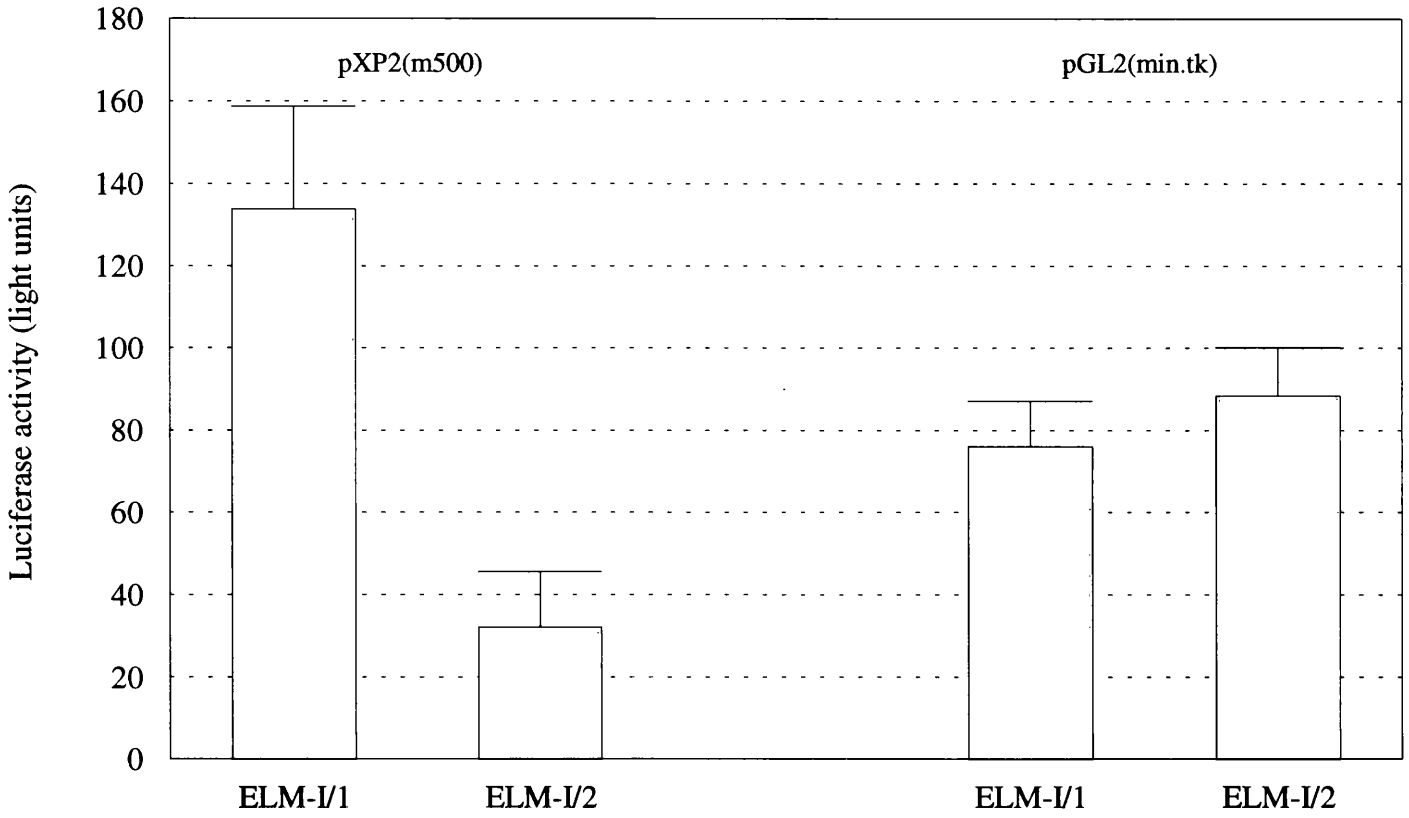
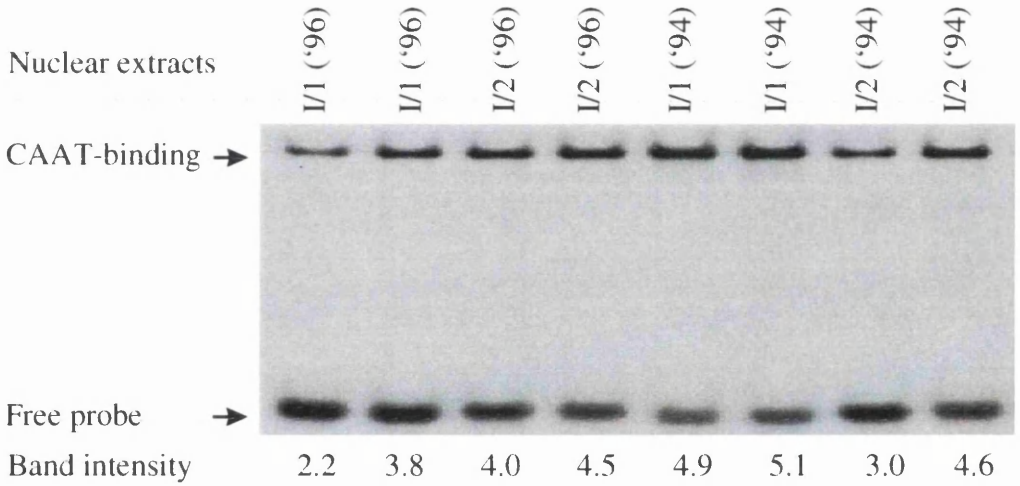


Figure 11.9. Quantitation of nuclear proteins utilising the CAAT oligonucleotide which binds to ubiquitous nuclear protein(s) in gel retardation assays.

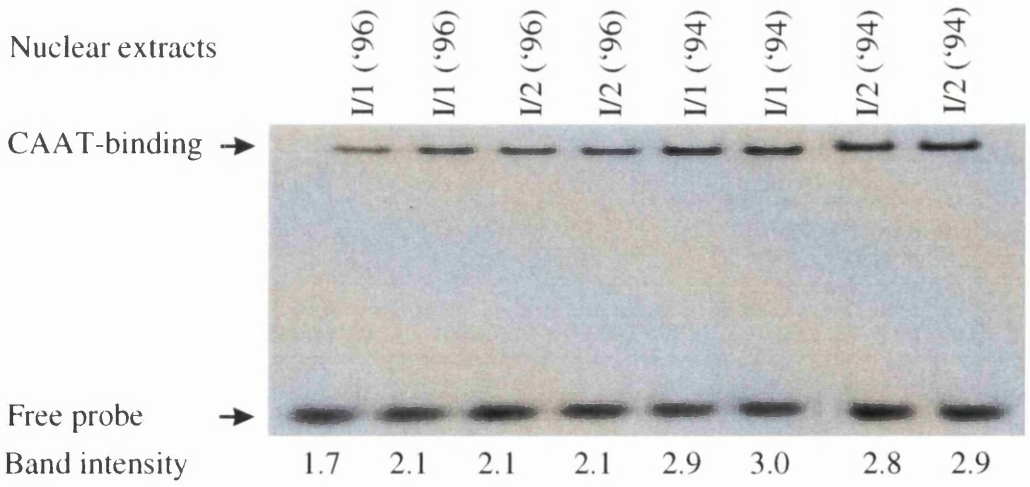
(a) Large scale nuclear protein extracts were prepared from ELM-I/1 and I/2 cells on two different occasions (the year of which, 1994 and 1996, are indicated in brackets). The protein concentrations were quantitated by the BCA assay (see Methods and Materials) followed by gel retardation analysis using the CAAT oligonucleotide (see Section 11.2). The intensity of the retarded bands was quantitated by densitometry. The calculated figures were shown below each lane, which were then used to further standardise the protein loadings.

(b) Confirmation of equal protein loadings after quantitation by the BCA assays and CAAT-binding assays (see above). The gel retardation assay using the CAAT oligonucleotide was performed as in (a) but using the amounts of extracts that were calculated to give the same CAAT-binding. The intensity of the bands, which was indicated below each lane, shows equal loadings from different protein preparations, and therefore the reliability of the quantitation results.

(a) Before correction for CAAT-binding activity



(b) After correction for CAAT-binding activity



but it seems that there were still differences in the CAAT-binding from various protein preparations. The retarded bands resulting from the CAAT-binding were subsequently scanned by densitometry and their optical density calculated. The differences in the loadings in Figure 11.9a were then further corrected according to the calculated optical density of the bands, and Figure 11.9b shows the CAAT-binding activity of nuclear proteins from various preparations after such correction. It can be seen from the quantitated CAAT binding activity that proteins were equally loaded in Figure 11.9b. The results of such quantitation were used in the subsequent Western blotting, footprinting and gel retardation analysis.

To be certain that the methods used for the large scale nuclear protein preparations can successfully extract the *Fli-1* protein from ELM-I/1 cells, the nuclear extracts were analysed by Western blotting using a *Fli-1*-specific antibody. 1μg and 10μg of the ELM-I/1 and I/2 nuclear extracts prepared in two separate occasions were used. It can be seen in Figure 11.10 that the antibody recognised the *Fli-1* protein in the ELM-I/1 nuclear extracts, and resulted in a doublet with the molecular weights of 48 kD and 51 kD. The intensity of the bands representing *Fli-1* further confirms the reliability of the protein quantitation.

11.3. *In vitro* DNase I footprinting analysis of the minimum *bcl-2* promoter region.

To determine the *Fli-1* activating sites within the minimum *bcl-2* promoter, *in vitro* DNase I footprinting studies were performed with various fragments of the region suitable for identifying any protein-binding sites (Figure 11.11a and 11.11b). The promoter fragments used in footprinting analysis were generated by PCR from mouse genomic DNA, which were subsequently cloned into the vector pCR-Script.Amp.(SK+) (Stratagene) (see Methods and Materials). By using probes 1 and 2 labelled on complementary strands of the minimum *Fli-1*-responsive *bcl-2* promoter region identified, six fooprints (FPs) were identified with both ELM-I/1 and I/2 nuclear extracts (Figure 11.11a). FP3 and FP4 were close to the top of the gels and were not resolved as clearly as the other FPs. In order to improve the resolution of FP3 and FP4, similar foorprinting reaction was performed with probe 2 but the sequencing gel was run much longer (Figure 11.11b).

Figure 11.12 shows the positions of these FPs alongside the sequence of the promoter fragment used. Four FPs (FP2, FP3, FP4 and FP6) contain potential binding sites for the *Ets* family of transcription factors. The two potential *Ets*-binding sites (EBS) in FP2 and FP4 are palindromically arranged. This is potentially interesting as it has been reported that, at least in some cases, efficient DNA-binding and transactivation by the *Ets* proteins require two EBS arranged in an inverted configuration, whereas the

Figure 11.10. Western blotting analysis of the presence of the *Fli-1* protein in the large scale nuclear protein preparations from ELM cells.

Large scale nuclear protein extracts were prepared from ELM-I/1 and I/2 cells on two different occasions (the year of which, 1994 and 1996, are indicated in brackets). The molecular weights are indicated.

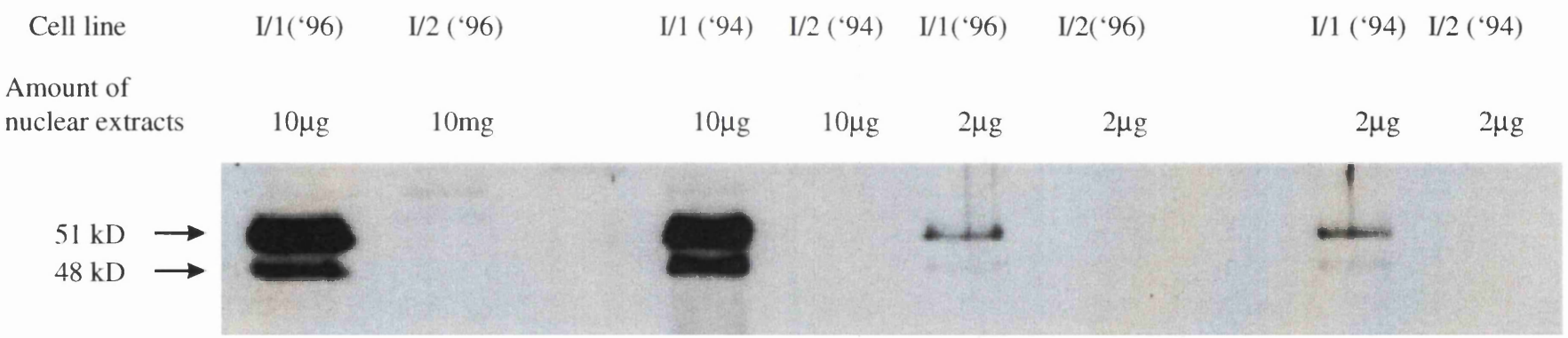


Figure 11.11 *In vitro* DNase I footprinting analysis of the minimum *bcl-2* promoter region. (a) Footprinting analysis using probes 1 and 2 as described below; (b) Similar footprinting analysis using probe 2 but the gel was run much longer in order to reveal the FP3 and FP4 more clearly.

Footprinting probes were prepared by 5'-end labelling (indicated by the star) of DNA restriction fragments with T4 polynucleotide kinase and [α-^{32}P]ATP, and isolated after secondary restriction digest (see Methods and Materials). DNA:protein binding reactions were carried out in the presence of poly(dI-dC)·poly(dI-dC) with the nuclear protein extracts from ELM-I/1 and ELM-I/2 cells. After partially digested with DNase I, the nucleic acid was purified and resolved by 6% denaturing polyacrylamide gel electrophoresis and autoradiography (see Methods and Materials). A control reaction (O) was included, where protein extracts were omitted. A+G chemical sequencing reactions (see Methods and Materials) of the probes, together with the position of the sequence relative to the transcription initiation site, are included. The positions of the footprints (FP1-FP6) referred to in the text are marked. The putative *Ets*-binding sites are underlined. The TATA, CAAT and Oct-1 motifs are also indicated.

The numbering of the sequence is based on the *bcl-2* genomic sequence published by Negrini *et al.* (1987).

Figure 11.11 (a)

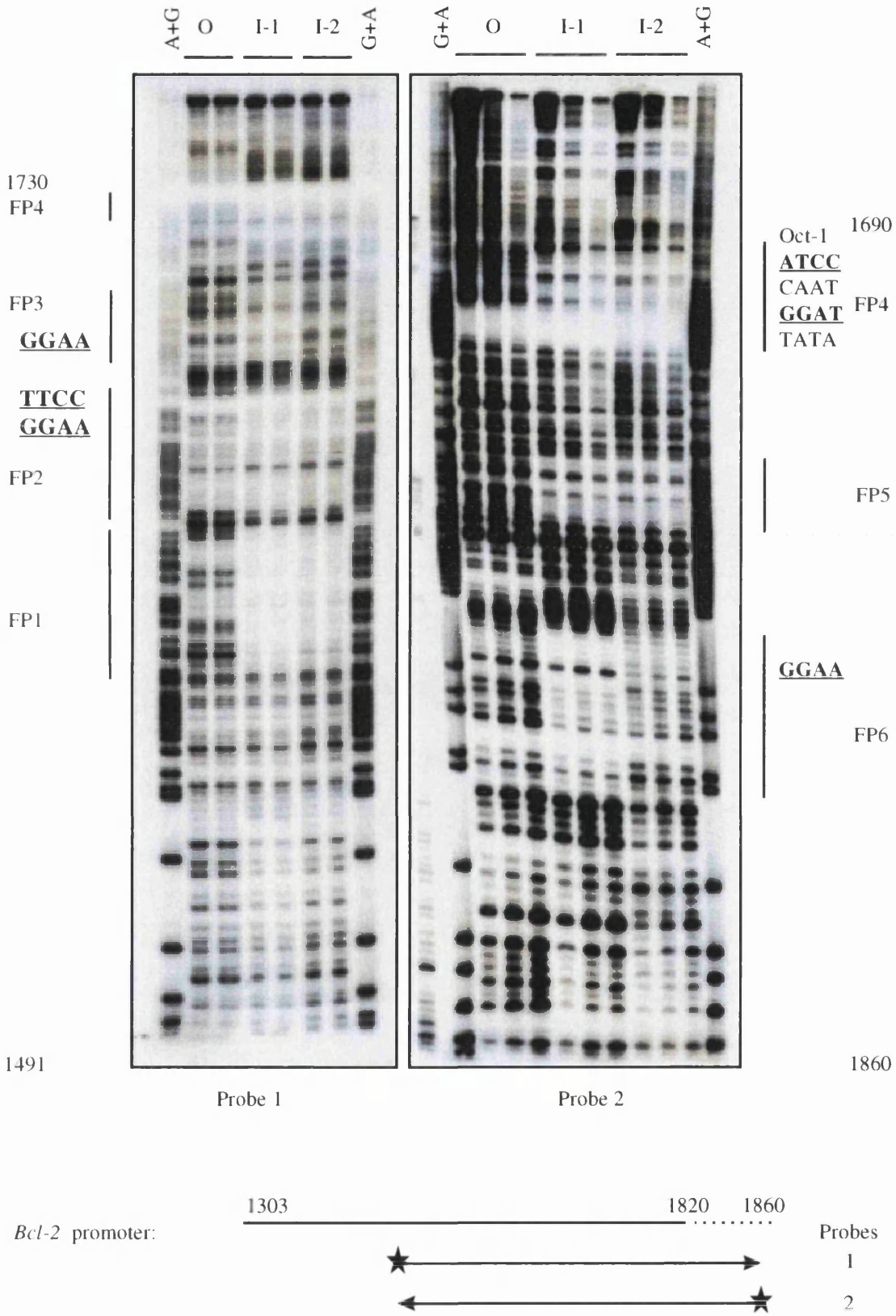
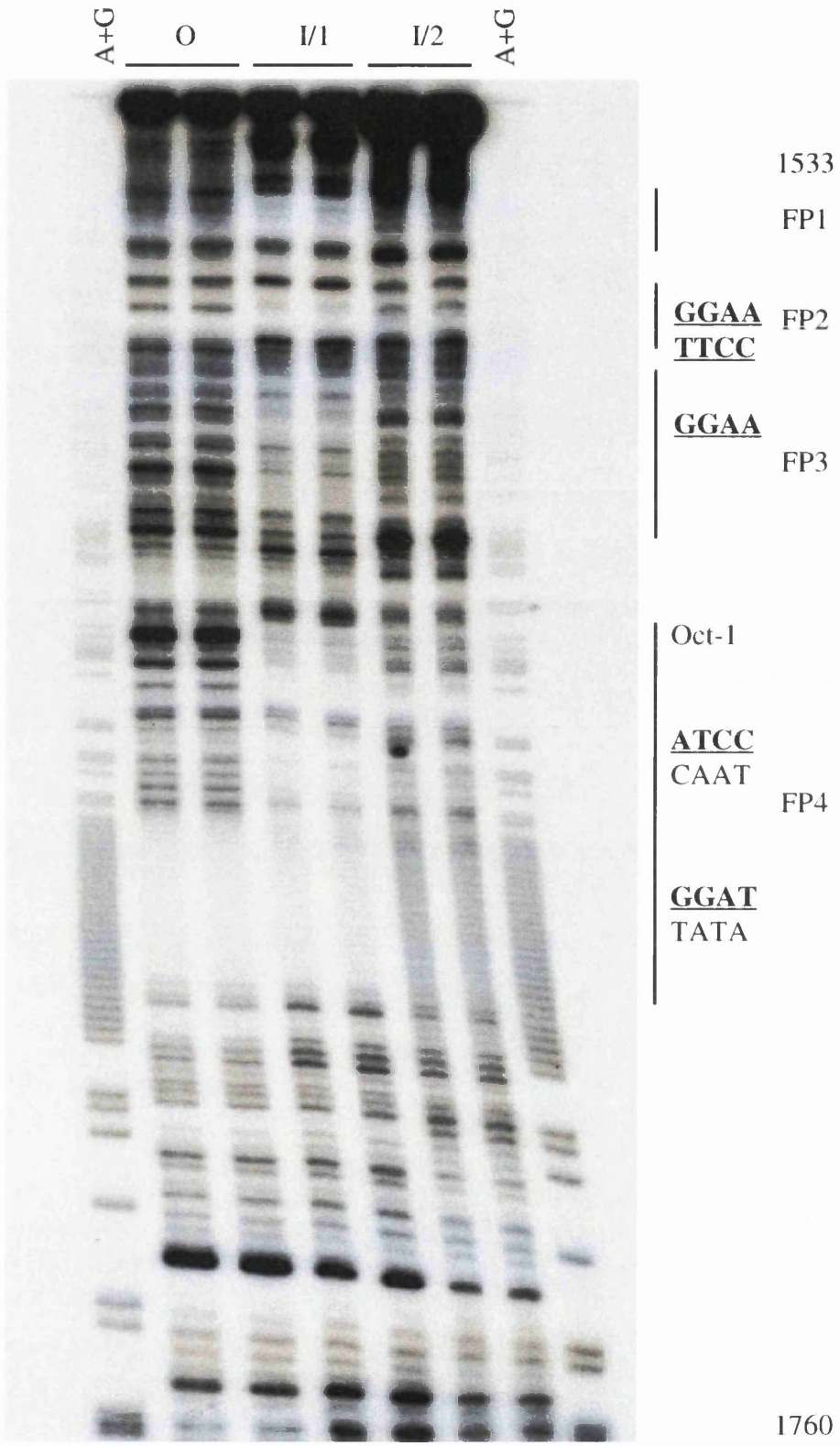


Figure 11.11 (b)[a]



Probe 2 (the longer run)

[a] See page 116 for the legend and Figure 11.11 (a) for details of the probe.

Figure 11.12 Positions of the six footprints (FP1-FP6, underlined) alongside the sequence of the *bcl-2* promoter fragment used in DNase I footprinting analysis. The potential binding *Ets*-binding sites (EBS) are boxed. The Oct-1, TATA and CAAT motifs are also indicated.

```
1491                                                        TAACTTCCTA

1501  GGTCGTCCCG CCTCTTCACC TTTCAGCATT GCGGAGGAAG TAGACTGATA
                                                  EBS1

1551  TTAACAAAGC TTAATAAATA ATGTACCTCA TGAAATAAAA AGCAGAAAGG
                    FP1                                   EBS2

1601  AATTTGAATA AAAATTTCCT GCATCTCATG CCAACGGGGA AACACCAGAA
        FP2          EBS3                      EBS4

1651  TCAAGTGTTC GGTGTAACTA AAGACACCCC TTCATCCAAG AATGCAAAGC
              FP3                                      Oct-1

1701  ACATCCAATA AAAGAGCTGG ATTATAACTA TTCTTTTTTT TTTTTTTTTC
    EBS5/CAAT       FP4 EBS6/TATA

1751  TTTCGGGGCC GTGGGGCGGG AGTCGGGACT TGAAGTGCCA TTGGTACCTG
                                                  FP5

1801  CAGCTTCTTT TCGGGGAAGG ATGGCGCAAG CCGGGAGAAC AGGGTATGAT
                    EBS7      FP6

1851  AACCGGGAGA
```

oligonucleotide containing the two EBS in the same orientation binds 10-fold less proteins (Seth *et al.*, 1993). FP6 is the most interesting because there is an obvious difference in the footprinting patterns between the ELM-I/1 and I/2 nuclear extracts and the fact that it contains a potential EBS.

11.4. Gel retardation analysis of the identified footprints in the minimum *bcl-2* promoter region.

11.4.1. Nuclear factor(s) in the ELM-I/1 and I/2 nuclear extracts can bind specifically to all the footprints.

In order to identify the nuclear factor(s) binding to FP1-FP6, gel retardation analysis was performed with the ELM-I/1 and I/2 nuclear extracts. The 5'-boundary of FP2 is somewhat ambiguous, and so two oligonucleotides (FP2L and FP2S) varying at 5'-end were synthesised and used in gel retardation analysis (see Methods and Materials). Figure 12.13 shows that nuclear factor(s) in the ELM-I/1 and I/2 nuclear extracts can bind to all the footprints. There was no significant difference in protein-binding between the two different forms of FP2 and, therefore, the longer version of FP2 (i.e. FP2L) was used in the subsequent gel retardation assays. Furthermore, the bindings of all FPs were specific as judged by self-competition experiments where a 250-fold excess of unlabelled FP oligonucleotides were used (Figure 11.13 and 11.14). FP3 and FP6 are more potentially interesting since, in each case, an additional band was observed with the ELM-I/1 nuclear extracts (each indicated by an arrow in the figure), which did not appear with the ELM-I/2 extracts. Competition between different FP oligonucleotides was tested and, in all cases, the ELM-I/1 and I/2 nuclear extracts were used for comparison, and the autorads were exposed for the same length of time in the same cassette. It can be seen from Figure 11.14 (a-f) that there were competitions between certain FP oligonucleotides for protein-binding in gel retardation assays (summarised in Table 11.1). For example, either FP1 or FP4 was able to compete with FP2 and, similarly, FP5 with FP4. It seems that FPs adjacent to one another (e.g. FP1 and FP2 or FP4 and FP5) are more likely to compete with each other for protein-binding.

11.4.2. None of the ets oligonucleotides, E74, Ets-3, Fli, PEA3 or PU.1, competes significantly with the six FPs for protein-binding.

Competition experiments using the ets oligonucleotides were also carried out. These ets oligonucleotides, including E74, Ets-3, Fli, PEA3 and PU.1, contain the binding sites for certain *Ets* proteins. In particular, the Fli oligonucleotide has been reported to be able to bind to the *Fli-1* protein in gel retardation analysis (Bosselut *et al.*, 1993).

Figure 11.13. Gel retardation analysis of nuclear protein-binding to oligonucleotides (FP1-FP6) from the six footprinted regions. Self-competition experiments using excess amount of unlabelled oligonucleotides were performed for testing binding specificity.

Gel retardation experiments with the ELM-I/1 and I/2 nuclear extracts were performed as described in Methods and Materials.The **labelled oligonucleotides** used in each reaction were shown above the autoradiographs, below which are the nuclear extracts used and the presence or absence of **unlabelled competitor oligonucleotides**: (-) indicates no competitor and (+) indicates self-competition using 250-fold excess of unlabelled oligonucleotides. Arrows indicate bands that are unique to ELM-I/1 nuclear extracts. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.

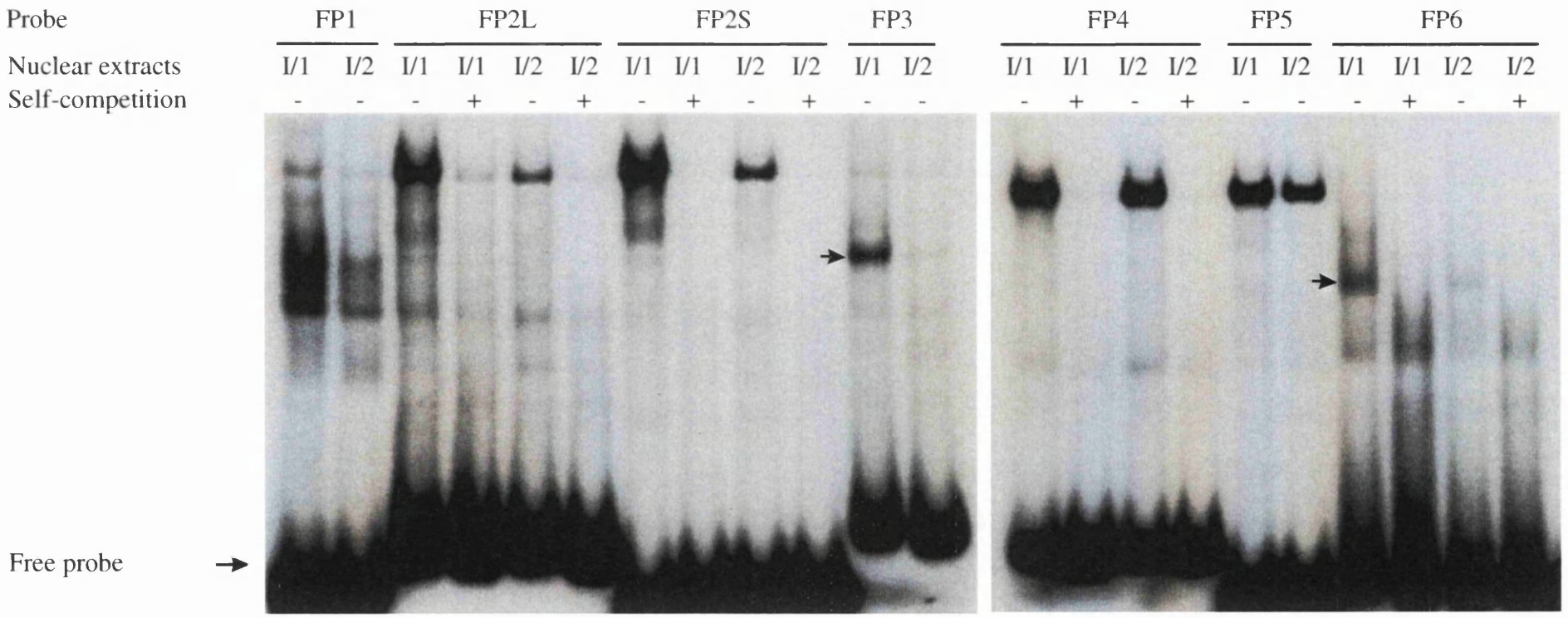


Figure 11.14. Gel retardation analysis of nuclear protein-binding to oligonucleotides (FP1-FP6) from the six footprinted regions: competitions between FPs and the established ets oligonucleotides, E74, Ets-3, Fli, PEA3 and PU.1.

Gel retardation experiments with the ELM-I/1 and I/2 nuclear extracts were performed as described in Methods and Materials. The **labelled oligonucleotides** and nuclear extracts used in each reaction are indicated. The **unlabelled competitor oligonucleotides** used are indicated above individual lane: (0) indicates no competitor; when competitors were included, 250-fold excess was used. Arrows indicate the bands that are unique to ELM-I/1 nuclear extracts. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.

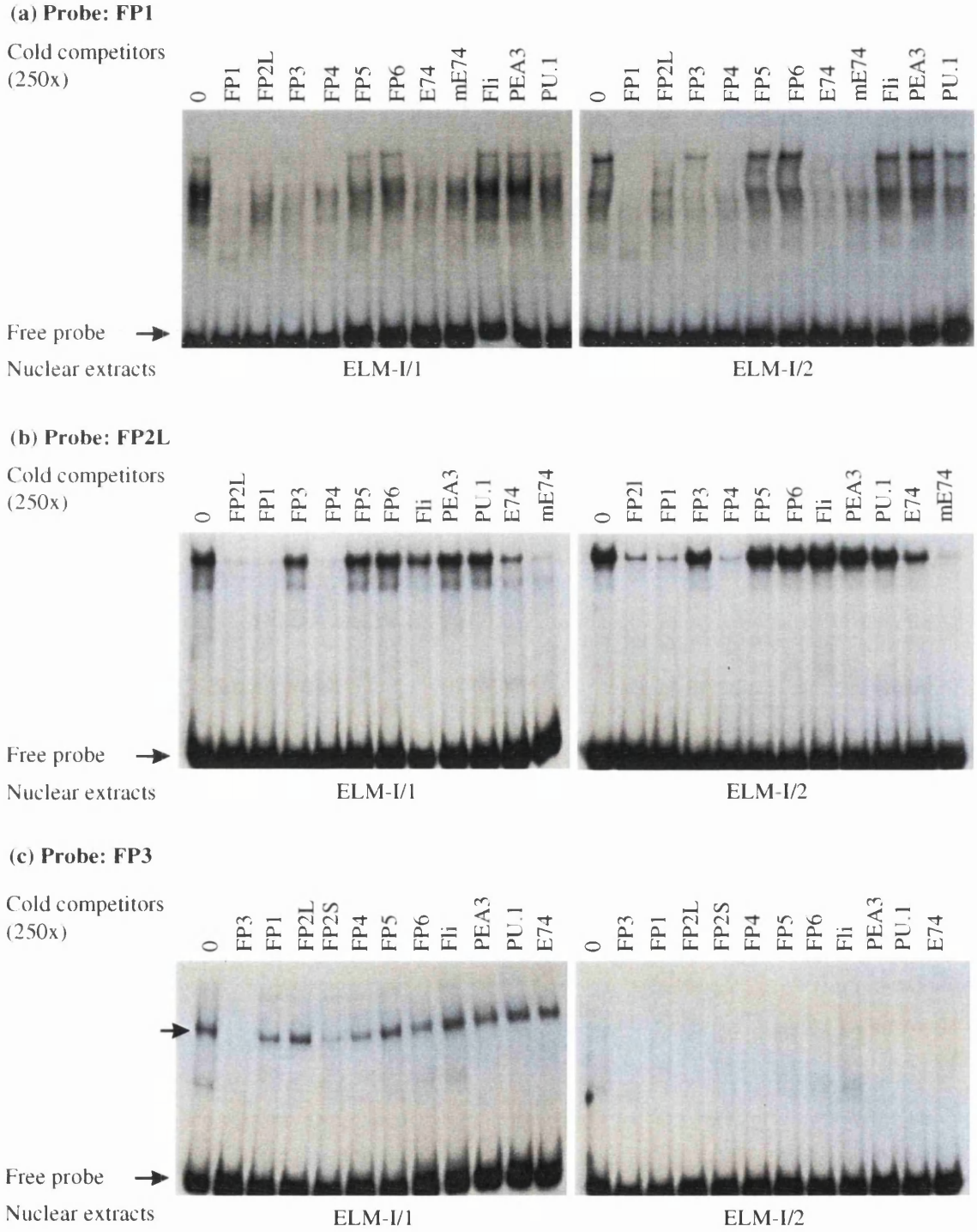


Figure 11.14. (Continued, see the previous page for the legend)

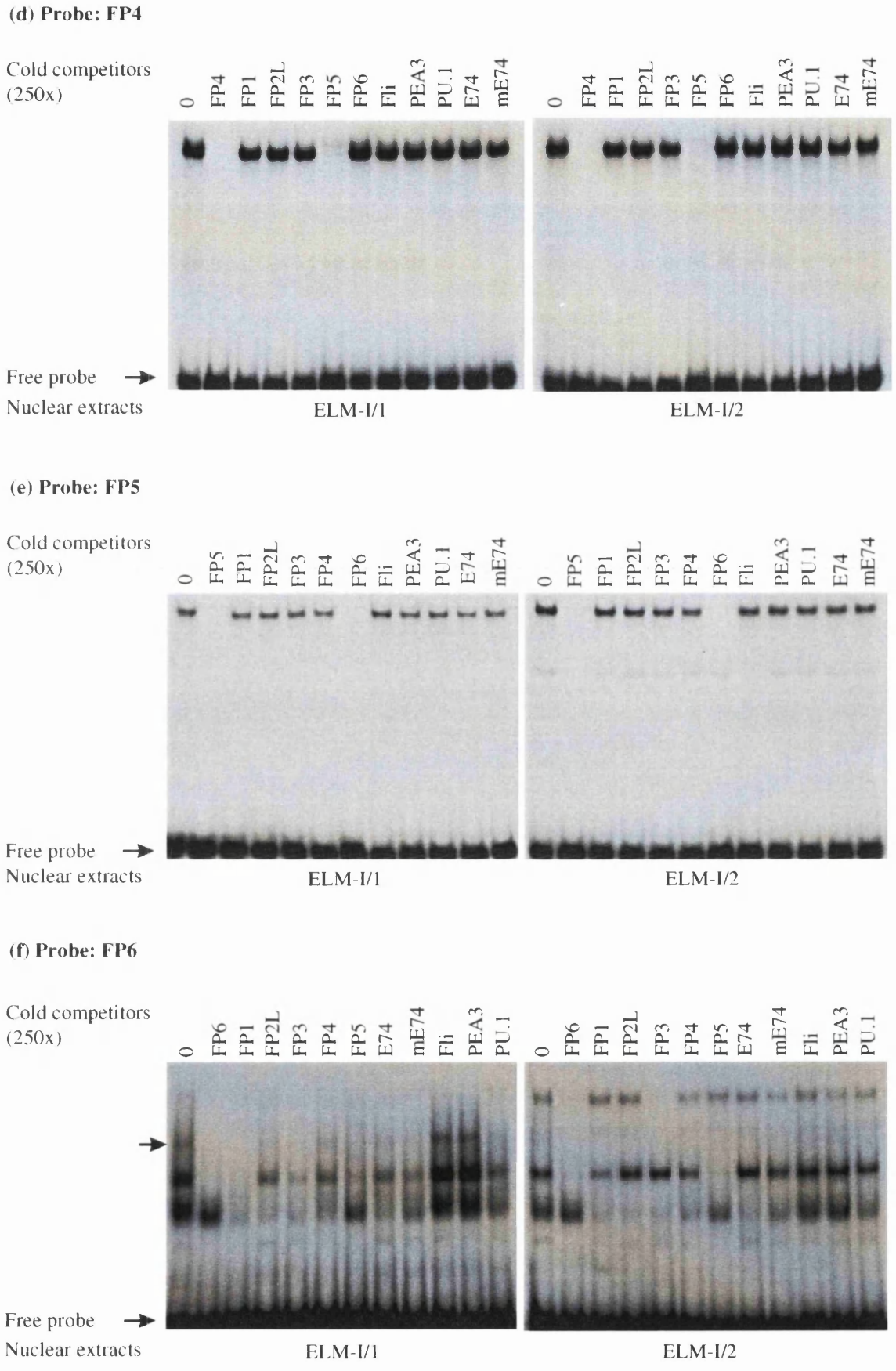


Table 11.1 Summary of gel retardation and competition analysis of the six footprint oligonucleotides (FP1-FP6). Note that FP1-FP6 in the first row indicates the probes used. FP1-FP6 along with other oligonucleotides in the first column are used as unlabelled competitors.

Probes	**FP1**	**FP2L**	**FP3**	**FP4**	**FP5**	**FP6**
Positions	1538-1582	1575-1631	1626-1688	1686-1736	1772-1807	1805-1840
Length	45 bp	57 bp	63 bp	51 bp	36 bp	36 bp
Potential Ets-sites	0	Ets② Ets③	Ets④	Ets⑤ Ets⑥	0	Ets⑦
Differential binding[a]	no	no	yes	no	no	yes
Potential interest[b]		*	***	*		***
FP1	+++	+++	-	-	-	+++/-
FP2L	++/-	+++	-	-	-	++/-
FP3	+++	-	+++	-	-	+++/-
FP4	+++/-	+++	+?	+++	+++/-	++/-
FP5	-	-	-	+++	+++	++/-
FP6	-	-	-	-	+++/-	+++/-
E74	++/-	+	-	-	-	+/-
mE74	++/-	+++	?	-	-	+/-
Fli	-	-	-	-	-	-
Pea3	-	-	-	-	-	-
PU.1	+/-	-	-	-	-	+/-

[a.] "Differential binding" indicates if there is any difference between the ELM-I/1 and I/2 nuclear extracts in binding to the footprint oligonucleotides in gel retardation analysis.
[b.] "Potential interest" grades the possibility of each footprint in light of *Fli-1* activation based on (1) if it contains potential *Ets*-binding sites and (2) if it displays differential binding to the ELM-I/1 and I/2 nuclear extracts.

However, none of these ets oligonucleotides, apart from E74, was able to compete with FP1-FP6 for protein-binding (Figure 11.14 (a-f)). E74 was able to compete with either FP1, FP2 or FP6, indicating that the *Ets* proteins might bind to these footprints. Consistent with this possibility is the fact that, in both FP2 and FP6, there are potential EBS. However, the mutated E74 oligonucleotide (mE74), in which the *Ets*-binding site is mutated, was also able to compete with FP1, FP2 and FP6, suggesting that the competition effects observed with E74 may be an artefact.

The lack of significant competition of E74 and Fli oligonucleotides with all the six FPs could be due to differences in binding affinity: if the *Ets* proteins bind to FPs with high affinity then it may be difficult to detect competition by E74 or Fli even when excess amount is used. To exclude this possibility, gel retardation analysis was performed using either E74 or Fli oligonucleotides as probes and the six FPs as competitors. The results showed that none of the FP was able to compete with E74 or Fli for protein-binding when the ELM-I/1 nuclear extracts were used (Figure 11.15). It is important to point out that although the presence of competition between the FPs and ets oligonucleotides may indicate the involvement of *Ets* proteins in DNA-binding, the lack of it does not necessarily exclude this possibility.

In summary, competition experiments using ets oligonucleotides do not provide a strong evidence for the involvement of *Ets* proteins in binding to the footprints (summarised in Table 11.1).

11.4.3. *In vitro* synthesised *Fli-1* protein can bind to the E74 and Fli oligonucleotides but none of the six FPs in gel retardation analysis.

As discussed above, although competition experiments have given negative results, they do not necessarily exclude the involvement of *Fli-1* in binding to the FPs. An alternative approach to demonstrate that *Fli-1* can bind to any of the FPs is to use *in vitro* synthesised *Fli-1* protein. For *in vitro* synthesis of *Fli-1*, the pRC-CMV-*fli-1* expression vector was used in a transcription and translation coupled rabbit reticulocyte system, and aliquots were used in Western blotting and gel retardation analysis. This was compared with aliquots of the "mock" transcription/translation (TNT) mix where the empty expression vector, pRC-CMV, was used.

Figure 11.16 shows the Western blotting analysis of the *in vitro* synthesised *Fli-1* protein, where the total cell lysates from ELM-I/1 cells were used as the positive control. It can be seen that the *Fli-1* protein in I/1 cells exists as a doublet with the molecular weights of 48 kD and 51 kD. In the transcription/translation mix where the *fli-1* expression vector was used, there were two weak bands migrating at the similar

Figure 11.15. Gel retardation analysis showing that none of the six footprint oligonucleotides (FP1-FP6) can compete with the labelled E74 (a) or Fli (b) oligonucleotides for binding to proteins in the ELM-I/1 nuclear extracts.

Binding of the E74 and Fli-1 oligonucleotides to the ELM-I/1 nuclear extracts was analysed by gel retardation aeesays in the presence or absence of the unlabelled FP1-FP6 oligonucleotides as competitors. The **unlabelled competitor oligonucieotides** used were indicated above individual lane: (0) indicates no competitor; when competitors were included, 250-fold excess was used. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.

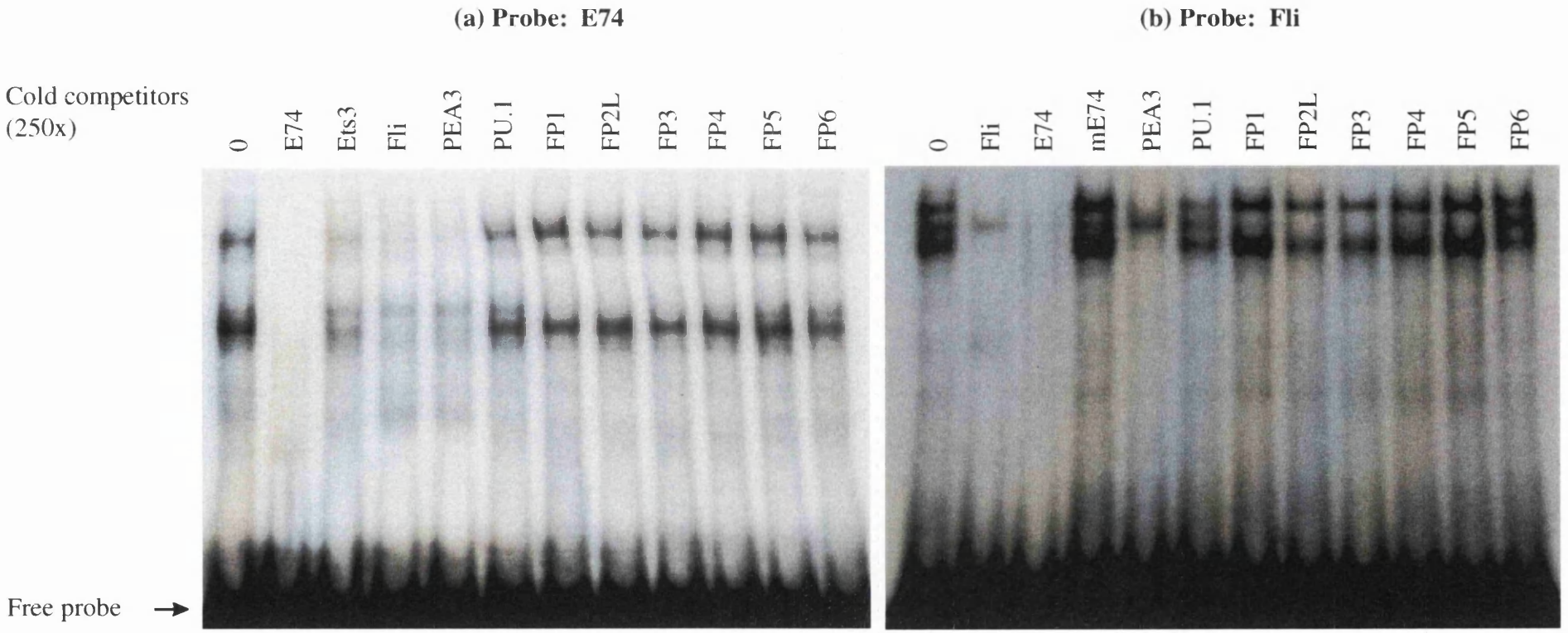
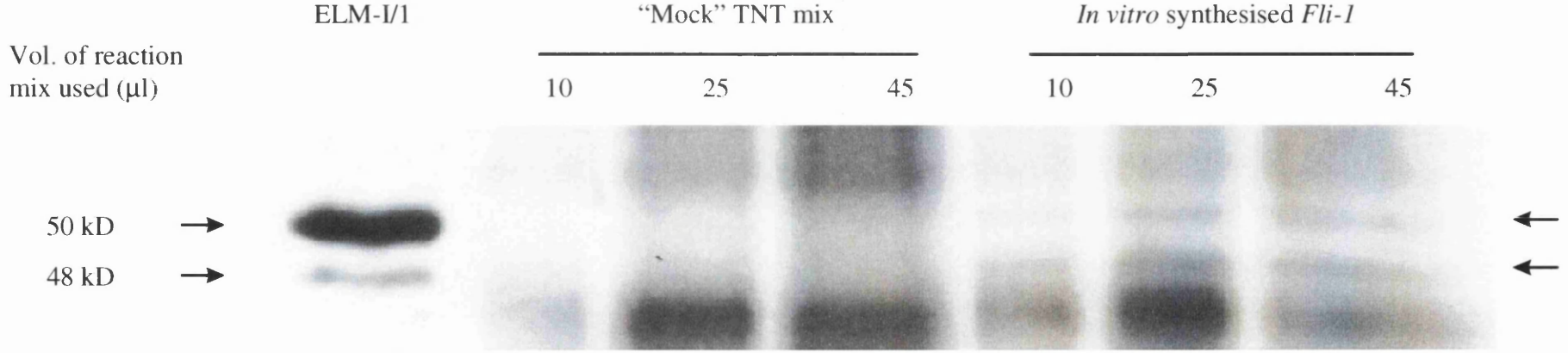


Figure 11.16. Western blotting analysis of the *in vitro* synthesised *Fli-1* protein.

In vitro synthesis of the *Fli-1* protein was carried out in a coupled transcription and translation (TNT) system from rabbit reticulocytes using a *Fli-1* expression vector (pRC-CMV-*Fli-1*) (see Methods and Materials). Aliquotes (10, 25 and 45µl) were denatured and then separated by SDS polyacrylamide gel electrophoresis followed by Western blotting analysis using a *Fli-1*-specific antibody (see Methods and Materials). As a negative control, a "mock" transcription/translation reaction was carried out with the empty expression vector, pRC-CMV, and aliquots (indicated as "Mock" TNT mix) analysed in parallel. The total cell lysate from ELM-I/1 cells was used as a positive control for the detection of *Fli-1* protein. The bands that represent *in vitro* synthesised *Fli-1* protein are indicated by arrows. The molecular weights are also indicated.



positions, which were not present in the "mock" transcription/translation mix where the pRC-CMV vector was used. This demonstrates that the transcription/translation system can produce the *Fli-1* protein *in vitro* with the correct molecular weight. However, the amount is poor compared to that present in ELM-I/1 cells.In order to demonstrate that the proteins synthesised in this way are functional in terms of their DNA-binding activity, gel retardation analysis was performed to test whether the *in vitro* synthesised *Fli-1* protein could bind to either E74 or Fli oligonucleotides as expected. It has been shown in Section 11.1.1 that a minimum tk promoter can be transactivated by *Fli-1* via the associated three copies of the E74 sequence. In addition, the Fli oligonucleotide has been reported to be able to bind to the *Fli-1* protein in gel retardation analysis (Bosselut *et al.*, 1993). Figure 11.17 shows that factors in the "mock" reaction mix were able to form complexes with both E74 (lane 1) and Fli oligonucleotides (lanes 7 and 8). The binding of E74 seemed to be specific as judged by the fact that it was competed by 250-fold excess of unlabelled E74 (lane 2), but not the mE74 oligonucleotide (lane 3). As mentioned before, the mE74 oligonucleotide is a mutated form of E74, where the core consensus *Ets*-binding site GGAA is mutated to CAA thereby abolishing the binding by the *Ets* proteins. The binding of the Fli oligonucleotide seemed to be non-specific as judged by the self-competition experiment (lane 9).

When the *Fli-1* transcription/translation mix was used, additional bands appeared with both E74 and Fli oligonucleotides. In the case of E74 (lane 4), three additional band (C, D and E) were detected as well as the two that were detected with the "mock" reaction mix (A and B) were detected as well as another three bands (C, D and E). The binding between the *in vitro* synthesised *Fli-1* protein and E74 oligonucleotide was specific, since the bands were competed by 250-fold excess of unlabelled E74 (lane 5) but not mE74 (lane 6). When the *Fli-1* TNT mix was incubated with the labelled Fli oligonucleotdies, there were two extra bands (G and H, in lanes 10 and 11) in addition to the one observed with the "mock" reaction mix (lanes 7 and 8), and the binding was also specific (lane 12). The reason for the existence of multiple bands is not clear. It is possible that the higher mobility complexes were due to the binding of degraded or incompletely synthesised *Fli-1* proteins. Alternatively, the lower mobility complexes may be the results of binding between the oligonucleotides and the *Fli-1* oligomers. Finally, the *Fli-1* protein may also form complexes with other proteins in the lysates, and different co-factors may be involved for different oligonucleotides. This can explain not only the existence of multiple bands but also why the mobility of protein:DNA complexes with the E74 and Fli oligonucleotides was different.

However, when the six FP oligonucleotides were used as probes in similar gel retardation assays, it was not able to reveal any binding between *in vitro* synthesised *Fli-1* and any of the FPs. Figure 11.18 shows that factor(s) in the "mock" reaction mix

Figure 11.17. Gel retardation analysis showing the binding of *in vitro* synthesised *Fli-1* protein to the E74 and Fli oligonucleotides.

In vitro synthesis of *Fli-1* protein was carried out in a coupled transcription and translation (TNT) system from rabbit reticulocytes using a *Fli-1* expression vector (pRC-CMV-*fli-1*) (see Methods and Materials). Aliquots (5µl or 10µl) were used directly in gel retardation analysis. As a negative control, "mock" transcription and translation reactions were carried out where the empty expression vector, pRC-CMV, was used. Gel retardation analysis was carried out as described in Methods and Materials.

The **unlabelled competitor oligonucleotides** used were indicated above individual lane: (0) indicates no competitor; when competitors were included, 250-fold excess was used. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiograph. Note that the free labelled Fli oligonucleotides had run off the bottome of the gel.

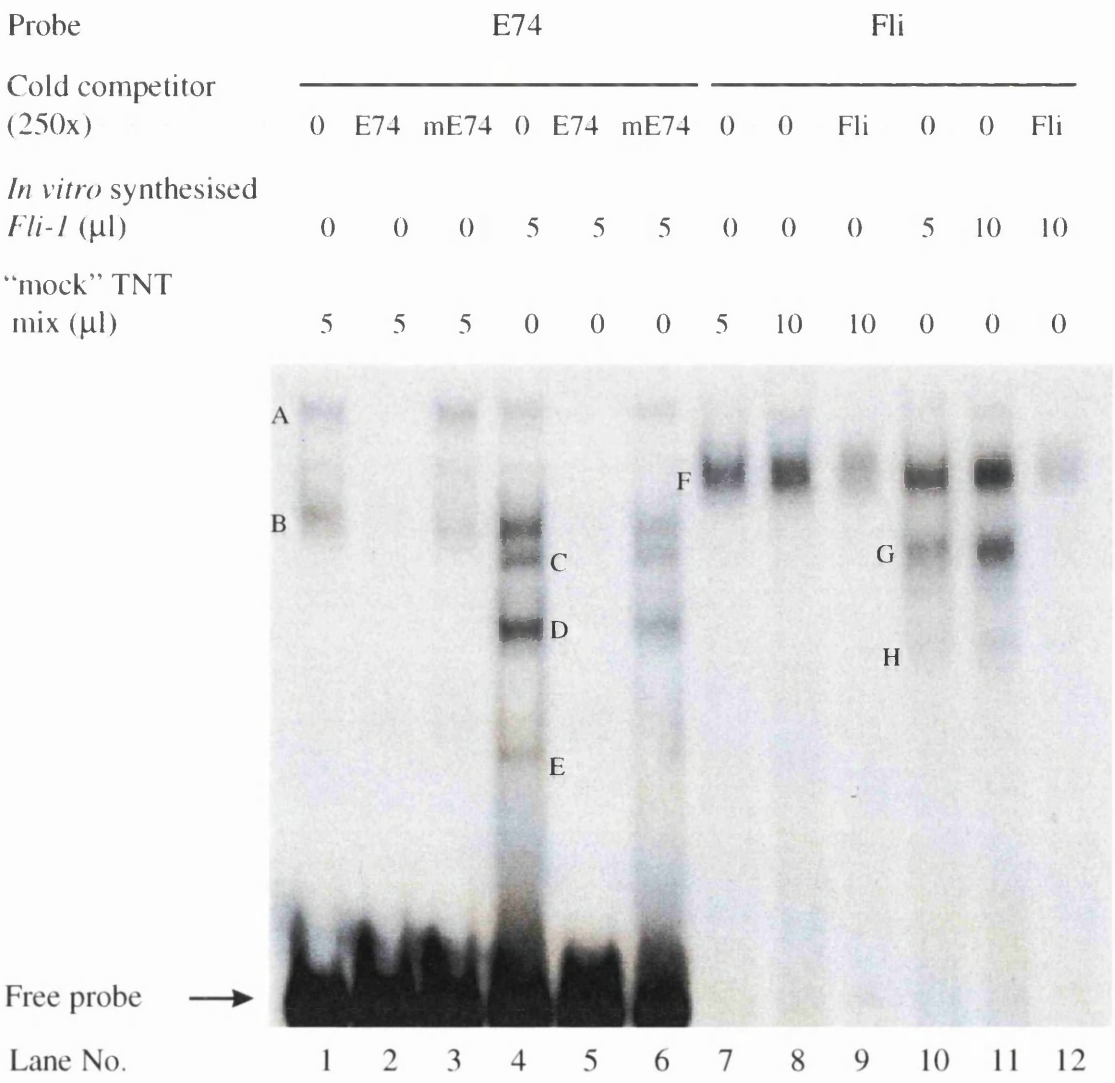
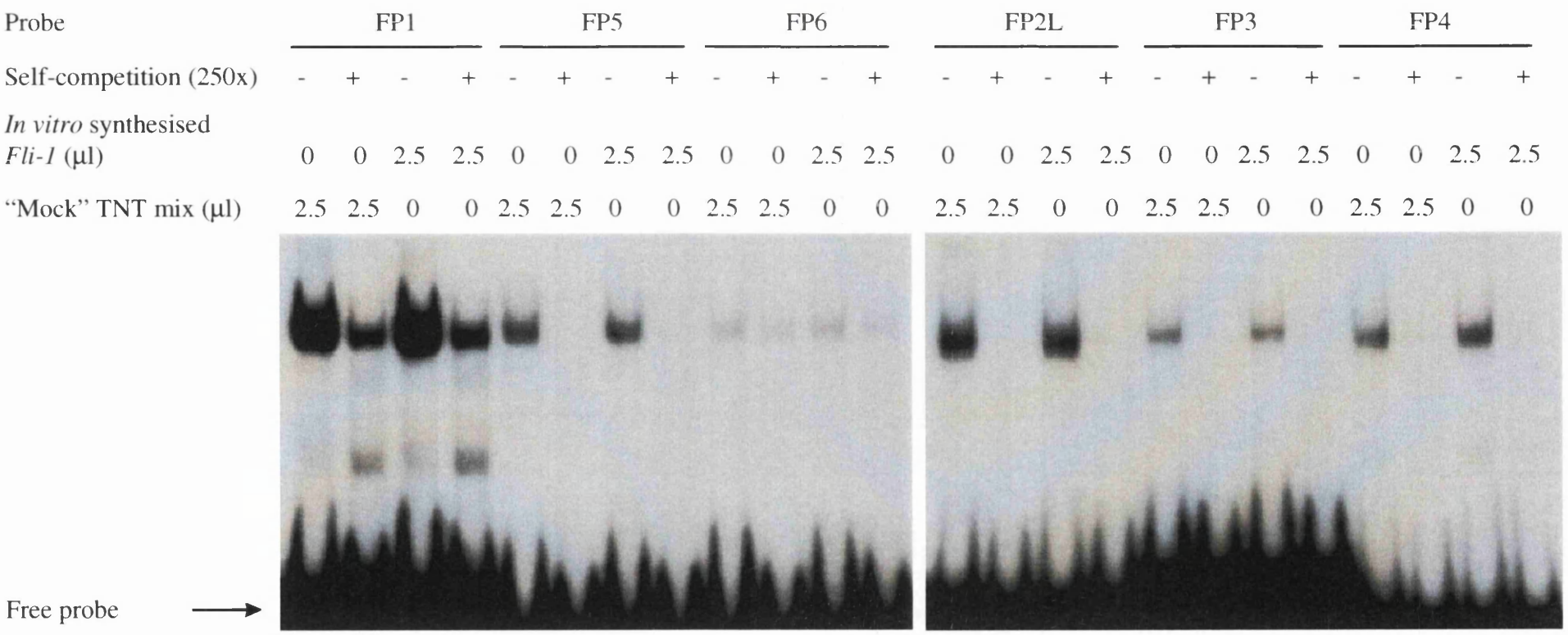


Figure 11.18. Gel retardation analysis showing that none of the six footprint oligonucleotides (FP1-FP6) can bind to the *in vitro* synthesised *Fli-1* protein.

Binding of *in vitro* synthesised *Fli-1* protein or the "mock" TNT reaction mix to FP1-FP6 was investigated by gel retardation assays as described in Figure 11.17 (also see Methods and Materials). The **labelled oligonucleotides** are shown above the autoradiographs. Self-competition using the **unlabelled competitor oligonucleotides** is indicated by (+); (-) indicates no competitor. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.



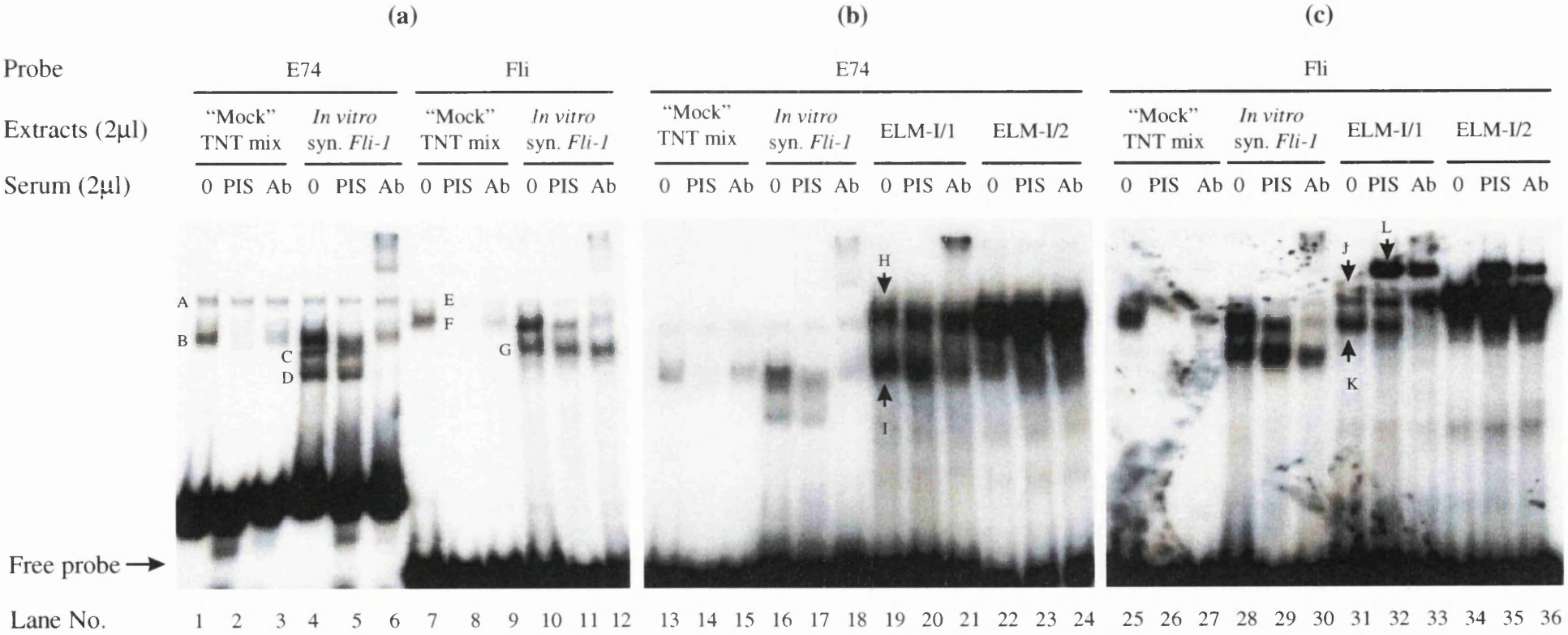
were able to bind to all of the FPs. *In vitro* sythesised *Fli-1* did not alter the intensity of the bands, nor did it give rise to any additional bands. It is possible that *in vitro* synthesised *Fli-1* could form complex(es) with FPs, but the complex(es) had the same mobility in gel retardation assays as those of FPs and some unknown factors in the "mock" reaction mix. This possibility can be tested in "supershift" analysis using a *Fli-1*-specific antibody (see Section 11.4.5). Alternatively, the data may also indicate that *in vitro* synthesised *Fli-1* protein cannot bind to any of the six FP oligonucleotides. However, it must be remembered that, in the absence of other co-factors, the DNA-binding activity of a protein produced in an unphosphorylated state *in vitro* could exhibit completely different properties as that in the context of a total nuclear protein extract prepared from growing cells. Indeed, a recurring theme with many *Ets* proteins is that their ability to recognise certain DNA sequences is altered by phosphorylation and protein:protein interaction with either *Ets* or non-*Ets* transcription factors (discussed in detail in Section 13.4). Therefore, the lack of binding between *in vitro* synthesised *Fli-1* and the FP oligonucleotides does not necessarily exclude the possibility that the *Fli-1* protein present in nuclear extracts can bind to the FPs.

11.4.4. The *Fli-1*-specific antiserum works in "supershift" analysis either when *in vitro* synthesised *Fli-1* or ELM-I/1 nuclear extracts are used.

To further investigate the involvement of the *Ets* proteins, in particular *Fli-1*, in binding to the FPs, "gel supershift" analysis was performed using the rabbit-anti-mouse *Fli-1* antiserum generated in our laboratory. In "gel supershift" analysis, if an antibody recognises the specific protein bound to a probe, then either the mobility of the protein:DNA complex(es) will be further retarded, or the intensity of the band(s) will be altered. In order to test whether or not the *Fli-1* antiserum is able to recognise *Fli-1* bound to oligonucleotides in gel retardation assays, *in vitro* synthesised *Fli-1* protein was incubated with either radiolabelled E74 or Fli in the presence of the antiserum. Pre-immune serum was included in the control reactions. Figure 11.19a shows that the *Fli-1* anti-serum was able to give rise to "supershifted" bands (lanes 6 and 12). Consequently, bands B, C, D and F, which either completely disappeared (C and D) or had decreased intensity (B and F), corresponded to the genuine *Fli-1*:DNA complexes. It is surprising that band G, which only appeared with *in vitro* synthesised *Fli-1*, was not affected by the antibody. It is possible that this band was due to the binding of the Fli oligonucleotide with a partially degraded *Fli-1* protein which has lost the epitope and therefore can not be recognised by the antibody. The "supershif" effects were due to the specific recognition of the *Fli-1* antibody and the *in vitro* synthesised *Fli-1* protein because: (1) "Supershift" was not detected with the pre-immune (lanes 5 and 11); and (2) The *Fli-1* anti-serum did not give rise to any "supershifted" bands in the control experiments where the "mock" translation mix was used (lanes 3 and 9). Taken together, the *Fli-1*

Figure 11.19. Analysis of the binding of *in vitro* synthesised *Fli-1* (a) and the *Fli-1* protein in ELM-I/1 nuclear extracts (b and c) to the E74 or Fli oligonucleotides by gel retardation assays using *Fli-1*-specific antiserum.

In vitro synthesised *Fli-1* protein, "mock" reaction mix, or the nuclear extracts from ELM-I/1 and I/2 cells were incubated with radiolabelled E74 or Fli oligonucleotides in the presence or absence of pre-immune serum (PIS) or *Fli-1* anti-serum (Ab), and then analysed by polyacrylamide gel electrophoresis (see Figure 11.17 and Methods & Materials). The **labelled oligonucleotides** were shown above the autoradiographs. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.



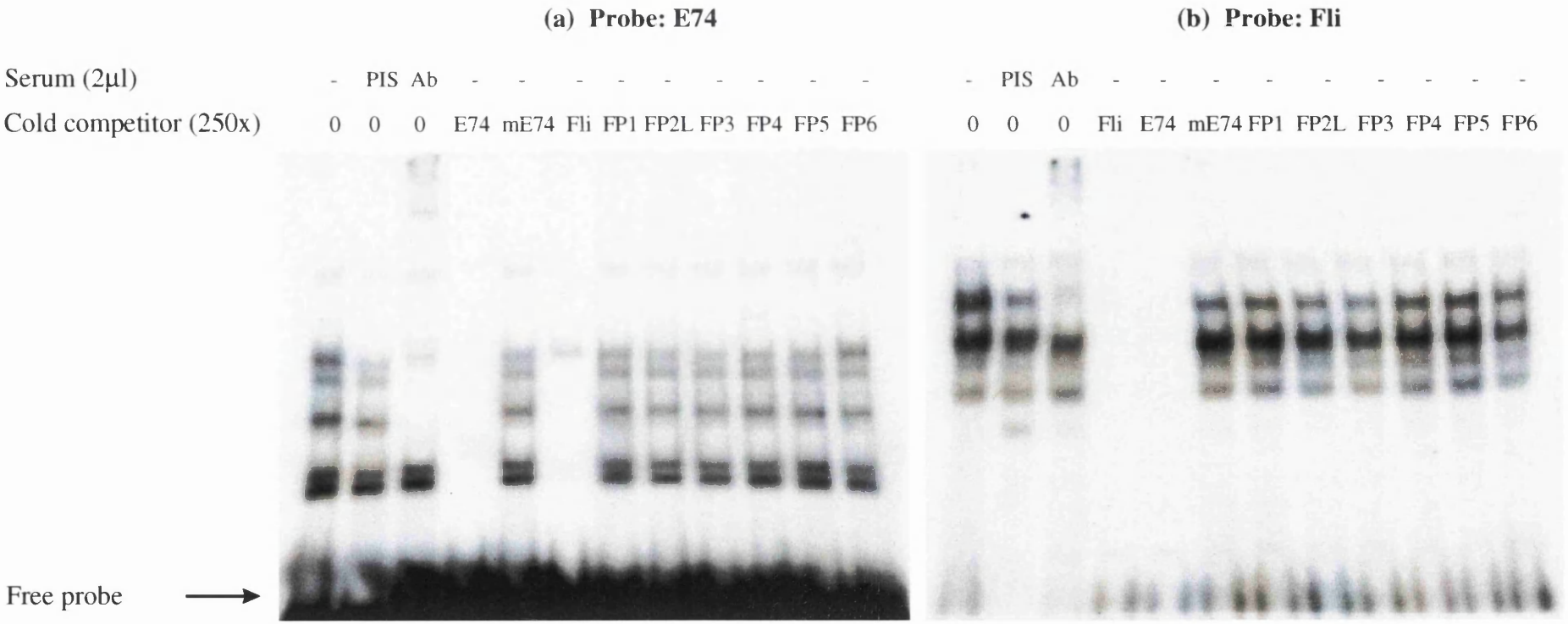
antiserum generated in our laboratory can recognise *in vitro* synthesised *Fli-1* protein in complexes with E74 or Fli oligonucleotides and give rise to "supershifted" bands in gel retardation assays.

To further determine whether the *Fli-1* anti-serum can also recognise the *Fli-1* protein present in the ELM-I/1 nuclear extracts, similar "supershift" assays were performed. *In vitro* synthesised *Fli-1* was used in parallel as the positive control. It can be seen in Figure 11.19b that the E74 oligonucleotide was able to bind to nuclear protein(s) in both ELM-I/1 and I/2 extracts (lanes 19 and 22). However, the *Fli-1* anti-serum gave rise to a "supershifted" band only when the ELM-I/1 extracts were used (lanes 21 and 24). The "supershift" resulted in a slight decrease in the intensity of band I, indicating that band I may represent the Fli-1:E74 complexes. This, together with the fact that ELM-I/1 but not I/2 cells express the *Fli-1* protein, indicates that the *Fli-1* anti-serum can indeed recognise the *Fli-1* protein in the ELM-I/1 nuclear extracts. Pre-immune serum did not has any effects on the banding patterns (lanes 20 and 23). Similar results were obtained with the Fli oligonucleotide (Figure 11.19c). It can be seen that nuclear factor(s) in both EM-I/1 and I/2 extracts were able to bind to the Fli oligonucleotide and gave rise to band J (lanes 31 and 34). However, factor(s) in the ELM-I/1 extracts also gave rise to an extra band (K) (lane 31), which was "supershifted" when the *Fli-1* anti-serum was included (lane 33). The anti-serum had no effects on band J with either ELM-I/1 or I/2 nuclear extracts (lanes 33 and 36). Although the pre-immune serum gave rise to band L, it did not have any effects on the intensity of bands J and K, nor did it cause any "supershift" (lanes 32 and 35). Band L may be a result of interaction of the Fli oligonucleotide with some non-specific factor(s) in the serum. Thus, the above evidence suggests that the "supershift" effects observed was due to the specific interaction between the *Fli-1* anti-serum and the *Fli-1* protein present in ELM-I/1 nuclear extracts. Therefore, the *Fli-1* anti-serum can not only recognise *in vitro* synthesised *Fli-1* protein but also the *Fli-1* protein present in ELM-I/1 nuclear extracts in gel retardation analysis, and give rise to "supershifted" bands.

Since it was thus established that *in vitro* synthesised *Fli-1* can bind to either E74 or Fli oligonucleotides in gel retardation assays, it was interesting to determine if any of the FPs can compete with E74 or Fli for *Fli-1* binding. As shown in Figure 11.20 that this is not the case. In the meanwhile, controls reactions including the *Fli-1* antibody were performed to reveal the *Fli-1* binding to either radiolabelled E74 or Fli, so were self-competition reactions and cross-competition between E74, mE74 and Fli.

Figure 11.20. None of the six footprint sequences (FP1-FP6) is able to compete with the E74 (a) or Fli (b) oligonucleotides for binding to the *in vitro* synthesised *Fli-1* protein.

Binding of *in vitro* synthesised *Fli-1* protein to the E74 (a) or Fli (b) oligonucleotides was carried out in the presence or absence of 250-fold excess of competitor, and then analysed by polyacrylamide gel electrophoresis (see Methods and Materials). The **unlabelled competitor oligonucleotides** used are indicated above each lane. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs. Note that the free Fli probe had run off the bottom of the gel. In "supershift" analyss, a *Fli-1*-specific antiserum was used (Ab), whereas the pre-immune serum (PIS) was used in the control reactions.



11.4.5. The *Fli-1*-specific antiserum does not reveal a direct interaction between *Fli-1* and any of the six FP oligonucleotides.

However, when "supershift" analysis was performed using ELM-I/1 and I/2 nuclear extracts and the six FP oligonucleotides as probes, we were not able to reveal any "supershifted" bands (Figure 11.21). This excludes the possibility that the complexes of FPs and *in vitro* synthesised *Fli-1* had the same mobility in gel retardation assays as those of FPs and some unknown factors in the "mock" reaction mix (see Section 11.4.3, in particular, Figure 11.18). The results imply that either the assay conditions are not suitable for detecting the interaction between *Fli-1* and FPs, or *Fli-1* does not bind to any of the six FPs (discussed in detail in Section 13.4). If the latter is true, then the transactivation of the partial *bcl-2* promoter by *Fli-1* may be due to some indirect effects (discussed in detail in Section 13.4).

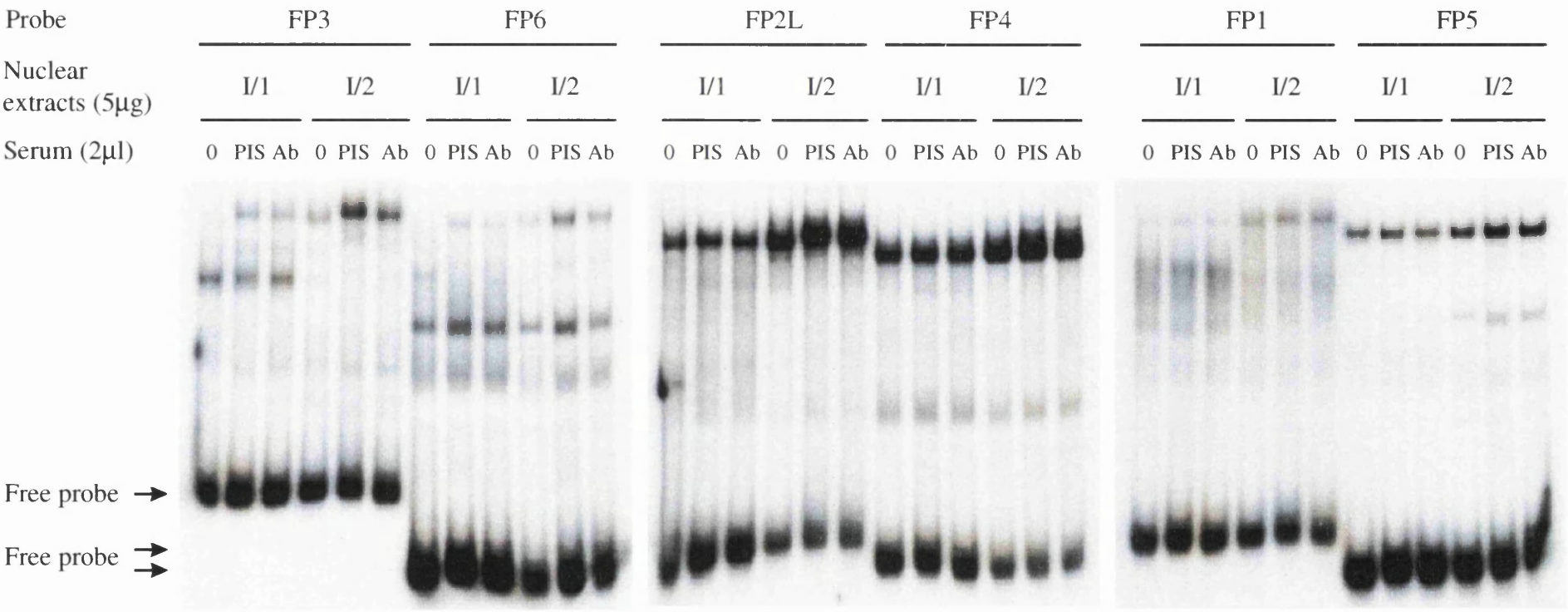
11.5. Conclusions.

Transient transfection assays have demonstrated that:
(1) *Fli-1* can transactivate a partial *bcl-2* promoter in a quail fibroblast cell line and a minimum promoter region has been defined;
(2) The *Bcl-2* promoter activity is higher in ELM-I/1 cells, which express high levels of *Fli-1* protein, than that in ELM-I/2 cells, which do not express *fli-1* at all.

The attempts to map the *Fli-1* activation site(s) within the minimum *bcl-2* promoter by DNA footprinting, gel retardation and "supershift" analysis have demonstrated that:
(1) Six footprints (FP1-FP6) have been identified within the minimum *bcl-2* promoter region with ELM-I/1 and I/2 nuclear extracts, two of which (FP3 and FP6) are different between the two cell lines;
(2) Nuclear factors in the ELM-I/1 and I/2 extracts can bind to all the six foorprints in a specific manner as judged by self-competition experiments;
(3) Nuclear factor(s) specifc in ELM-I/1 extracts can bind to the FP3 and FP6 oligonucleotides and give rise to bands that are not present with ELM-I/2 extracts;
(4) Out of all the ets oligonucleotides (E74, Ets-3, Fli, PEA3 and PU.1) tested, only E74 can significantly compete with some of the FPs (i.e. FP1, FP2 and FP6) for protein-binding. However, this competition may not be relevant as the mutated E74 oligonucleotide (mE74), in which the *Ets*-binding site is mutated, can can also compete in each case;
(5) None of the FP oligonucleotides can compete with labelled E74 or Fli for protein binding either when the ELM nuclear extracts or *in vitro* synthesised *Fli-1* were used;
(6) The E74 and Fli oligonucleotides, but none of the six FPs, can form complexes with the *in vitro* synthesised *Fli-1* protein;

Figure 11.21. Analysis of the binding of ELM nuclear extracts to the six footprint oligonucleotides (FP1-FP6) by gel retardation assays using *Fli-1*-specific antiserum.

Radiolabelled FP1-FP6 oligonucleotides were incubated with ELM-I/1 and ELM-I/2 nuclear extrcts in the presence or absence of pre-immune serum (PIS) or *Fli-1*- specific antiserum (Ab), and then analysed by polyacrylamide gel electrophoresis (see Figure 11.17 and Methods and Materials). The **labelled oligonucleotides** were shown above the autoradiographs. **Free probes**, which were not bound to any proteins in the extracts, are seen at the bottom of the autoradiographs.



(7) "Supershift" analysis using a *Fli-1*-specific antibody was not able to reveal the binding between *Fli-1* and any of the six FPs.

CHAPTER 12: Correlation of *Bcl-2* Up-regulation with Differentiation Arrest and Increased Tumourigenicity

12.1. Changes in the *bcl-2* expression in ELM cells undergoing erythroid differentiation induced by erythropoietin (Epo).

As mentioned in Chapter 5, *bcl-2* is the founding member of an expanding family of genes that are involved in the regulation of apoptosis in development *in vivo* and many tissue culture systems *in vitro* (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990). However, differentiation, in particular terminal differentiation, and apoptosis have been found to be related processes. Furthermore, the expression of *bcl-2* family of genes is developmentally regulated (Hockenbery *et al.*, 1991) and is also seen to be altered in differentiating cells (Hockenbery *et al.*, 1991; Gratiot-Deans *et al.*, 1993; Kozopas *et al.*, 1993; Lin *et al.*, 1993; Merino *et al.*, 1994; Marthinuss *et al.*, 1995; Zhang *et al.*, 1996). In light of these, it is intriguing that in ELM-I/1 cells, which are blocked in differentiation, the *bcl-2* expression is significantly up-regulated (Figure 10.1). It is envisaged that dysregulation of the *bcl-2* expression may contribute to the differentiation arrest in ELM-I/1 cells. As a starting point to address the possible role of *Bcl-2* in erythroid differentiation, changes in the *bcl-2* expression in ELM cells undergoing erythroid differentiation were studied.

It has been demonstrated in Chapter 9 that the *bcl-2* and *α-globin* expression increases in ELM-D cells upon stroma-withdrawal (Figure 9.6). It is possible that *Bcl-2* may be directly involved and play a positive role in erythroid differentiation. Alternatively, up-regulation of *bcl-2* expression may be a response of ELM-D cells to the withdrawal of survival and proliferation signals that are normally presented by the stromal cells. In order to distinguish these two possibilities in an attempt to address the roles of *Bcl-2* in erythroid differentiation, ELM-D cells were induced to differentiate with Epo in the presence of stromal cells. Furthermore, ELM-I cells were also induced to differentiate with Epo and changes in the *bcl-2* expression studied.

12.1.1. Changes in the *bcl-2* expression in ELM-D cells undergoing Epo-induced erythroid differentiation in the presence of stroma.

The ELM-D cells were maintained in the presence of the stroma and grown to sub-confluence. They were then trypsinised and 9 volumes of the ELM medium were added containing 1U/ml of Epo. Cells were harvested at 0, 6h, 1 day and 3 days post-induction. By shaking the flasks, ELM-D cells can be separated from the stroma, and it has been tested that ELM-D cells isolated in such a way contain negligible number of the stromal

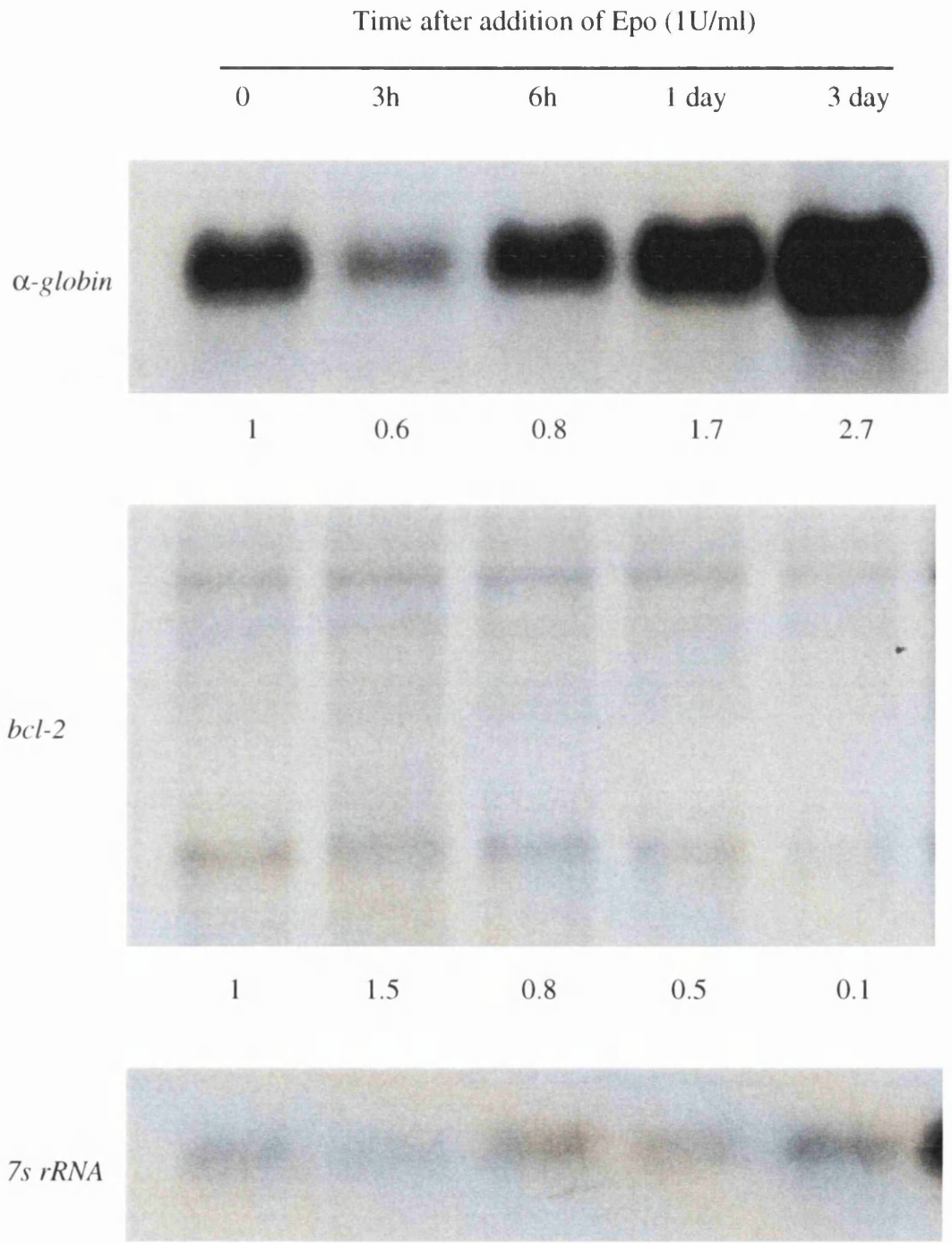
cells. The cell pellets of ELM-D cells harvested at day 1 and 3 were red, indicating that ELM-D cells, when grown in the presence of the stroma, can also differentiate in response to Epo. To quantitate this effect and analyse the changes in *bcl-2* expression during this process, total RNA was extracted and analysed by Northern blotting (see Methods and Matierials). The same blot was successively probed with a mouse *bcl-2* cDNA fragment and *α-globin* genomic fragment. RNA loading was assessed by stripping the blot of the radiolabelled probes and reprobing it with a 7s *rRNA* genomic fragment.

It can be seen from Figure 12.1 that, in response to Epo in the presence of stroma, ELM-D cells undergo erythroid differentiation, as indicated by an increase in the *α-globin* mRNA,. The increase in the *α-globin* expression reached approximately 3 fold at day 3 post-induction. During this process, the *bcl-2* mRNA decreased and, at day 3 post-induction, it was only 10% as the original level.

Although ELM-D cells can undergo erythroid differentiation in response to either stroma-withdrawal or Epo-addition in the presence of stromal cells, the molecular events were not entirely the same. First of all, it seems that ELM-D cells, in response to Epo in the presence of stromal cells, did not differentiate as well as in response to stroma-withdrawal. In the former situation, the *α-globin* mRNA increased to about 3 fold at day 3 post-induction by Epo (Figure 12.1); while in the latter, the *α-globin* expression increased to about 11 fold at day 3 after stroma-withdrawal (Figure 9.6). This confirms the earlier discovery (Chapter 9.5) that the stroma is able to block differentiation of the ELM cells, and further indicates that Epo can, to some extent, override this blockage. Secondly, the *bcl-2* expression underwent a transient increase followed by a later decline in ELM-D cells upon stroma-withdrawal (Figure 9.6); while the *bcl-2* expression decreased in ELM-D cells in response to Epo (Figure 12.1). It is possible that the initial increase in *bcl-2* expression is a response of ELM-D cells to the removal of survival and proliferation signals that are normally provided by the stromal cells. This effect can be so dramatic that it masks the decrease in *bcl-2* expression associated with erythoid differentiation. Alternatively, it is also possible that the population of cells with increased *bcl-2* expression upon stromal withdrawal was distinctive to those that underwent erythroid differentiation and had decreased levels of *bcl-2* expression. No matter what the explanation is, one thing that seems to be clear is that there are multiple cellular events in ELM-D cells upon stroma-withdrawal and the molecular changes (e.g. in the *bcl-2* expression) observed are a result of the combined effects. On the other hand, the cellular events of ELM-D cells in response to Epo are much simpler. Finally, as mentioned in Chapter 9.5, the observation that, upon stroma-withdrawal, ELM-D cells differentiate much better in 8% than 16% serum, but the increase in *bcl-2* expression was less in 8% serum (Figure 9.6 and 9.7), also points out

Figure 12.1. Changes in the α-*globin* and *bcl-2* mRNA expression of ELM-D cells in response to erythropoietin (Epo) in the presence of the stroma.

The ELM-D cells were cultured on the stroma in the presence of Epo. At 0, 6h, 1 day and 3 days post-induction, ELM-D cells were shaken off from the stroma, total RNA extracted and analysed in Northern blotting. The figures below the lanes are the relative levels of gene expression after calibration with the *7s rRNA* loading control.



that the *bcl-2* up-regulation may not be associated with erythroid differentiation. Therefore, the decrease in *bcl-2* expression observed in ELM-D cells in response to Epo in the presence of stroma can be regarded as a direct correlation with erythoid differentiation.

12.1.2. Changes in the *bcl-2* expression in ELM-I cells undergoing Epo-induced erythroid differentiation.

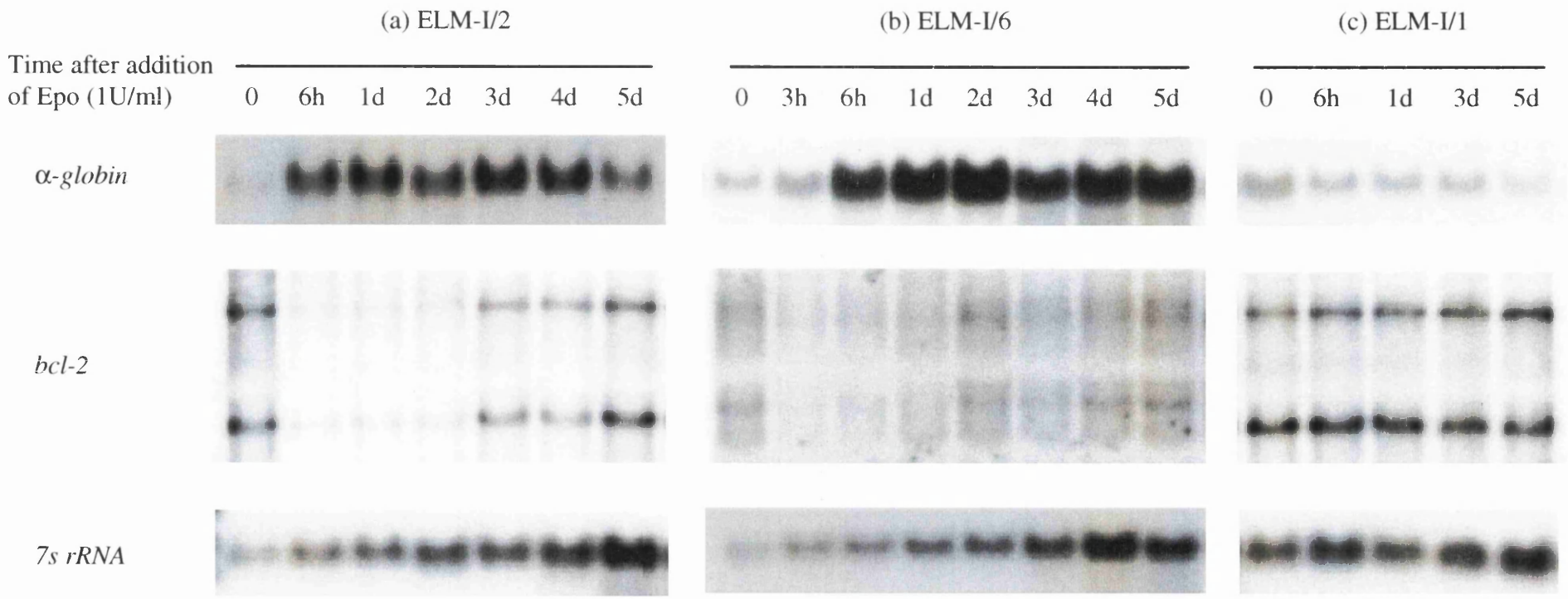
Similar induction experiments were carried out in ELM-I cells and gene expression studied. In contrast to ELM-I/1 cells, the cell pellets of ELM-I/2 and I/6 cells harvested at day 3 post-induction were red, indicating that ELM-I/2 and I/6 but not I/1 cells differentiate in response to Epo. To quantitate this effect and analyse the changes in *bcl-2* expression during this process, total RNA was extracted and analysed by Northern blotting as described above (see Section 12.1.1).

Figure 12.2 (a and b) shows that, in response to Epo, both ELM-I/2 and I/6 cells displayed an increase in the *α-globin* expression, which occurred as early as 6h post-induction. In the meanwhile, the *bcl-2* expression underwent a transient decrease followed by a later increase. The later increase in *bcl-2* expression occurred at day 2 post-induction, presumably at a time when the cells had underwent full differentiation. On the contrary, ELM-I/1 cells did not differentiate in response to Epo as indicated by the lack of increase in the *α-globin* expression (Figure 12.2c). Moreover, the *bcl-2* expression did not change at all during the period of experiment.

The above evidence suggests that there seems to be a correlation between the changes in the *bcl-2* expression and erythroid differentiation. Quantative changes in the expression of *bcl-2* and related genes have also been reported in other differentiation systems (Hockenbery *et al.*, 1991; Gratiot-Deans *et al.*, 1993; Kozopas et al., 1993; Lin et al., 1993; Merino *et al.*, 1994; Marthinuss *et al.*, 1995; Zhang *et al.*, 1996) and have been suggested to be necessary for cell differentiation. In ELM cells, it is possible that the early decrease in *bcl-2* expression is necessary for the cells to initiate differentiation program, in which case a constitutive up-regulation of the *bcl-2* expression, as seen in ELM-I/1 cells, may be responsible for differentiation arrest. Alternatively, changes in the *bcl-2* expression may be just a consequence of differentiation.

It is intriguing that, in ELM-D cells undergoing Epo-induced erythroid differentiation in the presence of stroma, the decrease in *bcl-2* expression was not followed by a later increase as observed with ELM-I/2 and I/6 cells. It is possible that the stromal cells may be able to block the increase in *bcl-2* expression. This hypothesis is consistent with that

Figure 12.2. Changes in the α-*globin* and *bcl-2* mRNA expression of ELM-I/2 (a), I/6 (b) and I/1 (c) cells in response to erythropoietin (Epo).



fact that, upon stroma-withdrawal, there was an increase in the *bcl-2* expression in ELM-D cells.

In summary, the *bcl-2* expression is decreased in ELM-D cells undergoing Epo-induced erythroid differentiation in the presence of the stroma. On the other hand, in the differentiating ELM-I/2 and I/6 cells in response to Epo, the *bcl-2* expression undergoes an early decline followed by a later increase. The *bcl-2* expression in ELM-I/1 cells, which express high levels of *bcl-2* and are blocked in differentiation, is not affected by the presence of Epo.

12.2. *Bcl-2* up-regulation and differentiation arrest in ELM-I cells.

In order to test whether the early decline in *bcl-2* expression in differentiating ELM cells is necessary and if constitutive up-regulation of *bcl-2* is responsible for differentiation arrest in ELM-I/1 cells, the *bcl-2* expression vector, pSFFV(*neo-bcl-2*), was stably transfected into ELM-I/2 and I/6 cells which do not express high level of *bcl-2*. The vector contains the full length cDNA of *bcl-2* and the neomycin-resistant gene driven by the promoter in the LTR of spleen focus-forming virus (SFFV), and has been reported to achieve high levels of *bcl-2* expression when transfected into cells (Bissonnette *et al.*, 1992). The control vector, pSSFV(*neo*), was used in parallel as a control for any artefacts that can be caused by transfection and the subsequent drug selection. Transfection was carried out by electroporation and neomycin-resistant clones were isolated (see Methods and Materials).

The efficiency of stable transfection (EST) can be calculated as the number of clones obtained in drug selection divided by the total number of cells seeded. The EST can be used not only as a measure of the transfection efficiency, but also as a way to detect any growth advantages or disadvantages of the cells caused by introducing the transgene. The EST of ELM-I/2 and I/6 cells when using the vectors pSSFV(*neo*) or pSSFV(*neo-bcl-2*) is shown in Table 12.1. It can be seen that there is no dramatic difference in the EST when using pSSFV(*neo*) and pSSFV(*neo-bcl-2*), and this is the case for both ELM-I/2 and I/6 cells, indicating that over-expression of *bcl-2* does not confer any growth advantages or disadvantages to the cells.

Table 12.1. Comparison of the efficiency of stable transfection of ELM-I/2 and I/6 cells when using either pSSFV(*neo*) or pSSFV(*neo-bcl-2*). The figures are results of three independent experiments.

	pSSFV(*neo*)	pSSFV(*neo-bcl-2*)
ELM-I/2	0.45%	0.31%
ELM-I/6	0.05%	0.02%

The expression of the *bcl-2* transgene at the mRNA and protein levels in individual clones was analysed by Northern and Western blotting analysis, respectively (see Methods and Materials). It can be seen from Figure 12.3 and 12.4 that high levels of *bcl-2* expression was achieved in ELM-I/6 and I/2 transfectants at both mRNA and protein levels. The top two bands on the Northern blots (Figure 12.3a and 12.4a) reflect the two isoforms of endogenous *bcl-2* mRNA, which are 7.5kb and 2.4kb, respectively. The band below is the exogenously expressed *bcl-2* mRNA. It can be seen that the level of exogenous *bcl-2* mRNA was expressed at a much higher level than that of the endogenous transcripts, and this was consistent with the expression of *Bcl-2* protein. Figure 12.3b and 12.4b shows that the clones that expressed exogenous *bcl-2* mRNA also expressed the *Bcl-2* protein at a level much higher than that in ELM-I/1 cells. The *Bcl-2* protein in the untransfected ELM-I/6 and I/2 cells was not readily detectable using this antibody, and those clones that did not express exogenous *bcl-2* mRNA did not express *Bcl-2* protein at a detectable level, as seen in the parental cells.

Clones that express high levels of exogenous *Bcl-2* protein were used in the differentiation induction experiments to see whether constitutive up-regulation of *bcl-2* is sufficient to induce differentiation arrest. Clones that did not express exogenous *bcl-2* at neither mRNA nor protein levels were used in parallel as controls. Cells were treated with 1U/ml of Epo and harvested at day 3 post-induction for analysis of *α-globin* mRNA induction as described previously (see Section 12.1.1). It can be seen from Figure 12.5 (a and b) that all clones, apart from one (i.e. 6C2), were still able to differentiate in response to Epo, as indicated by a significant increase in the *α-globin* mRNA expression. Although there was variation between clones in their differentiation capacity, with some clones having higher *α-globin* induction than the others, this variation showed no correlation with the level of *bcl-2* expression.

It can be concluded that the decline in *bcl-2* expression is not necessary for erythroid differentiation, and constitutive up-regulation of the *bcl-2* expression on its own is not sufficient to block differentiation in ELM-I/2 and I/6 cells and thus cannot explain the differentiation arrest in I/1 cells. If *Bcl-2* is involved in differentiation arrest in ELM-I/1 cells at all, other co-factors are required (discussed in detail in Section 13.3.1).

12.3. Bcl-2 up-regulation and increased tumorigenicity of ELM-I cells.

It has been noted that the original ELM tumour can be passaged *in vivo* and that both ELM-D and ELM-I/1 cells are tumorigenic (Itoh *et al.*, 1988). However, ELM-I/1 cells, which have constitutive up-regulation of *fli-1* and *bcl-2* expression, display the highest tumorigenicity in the ELM system (Nibbs *et al.*,1993). In order to test whether

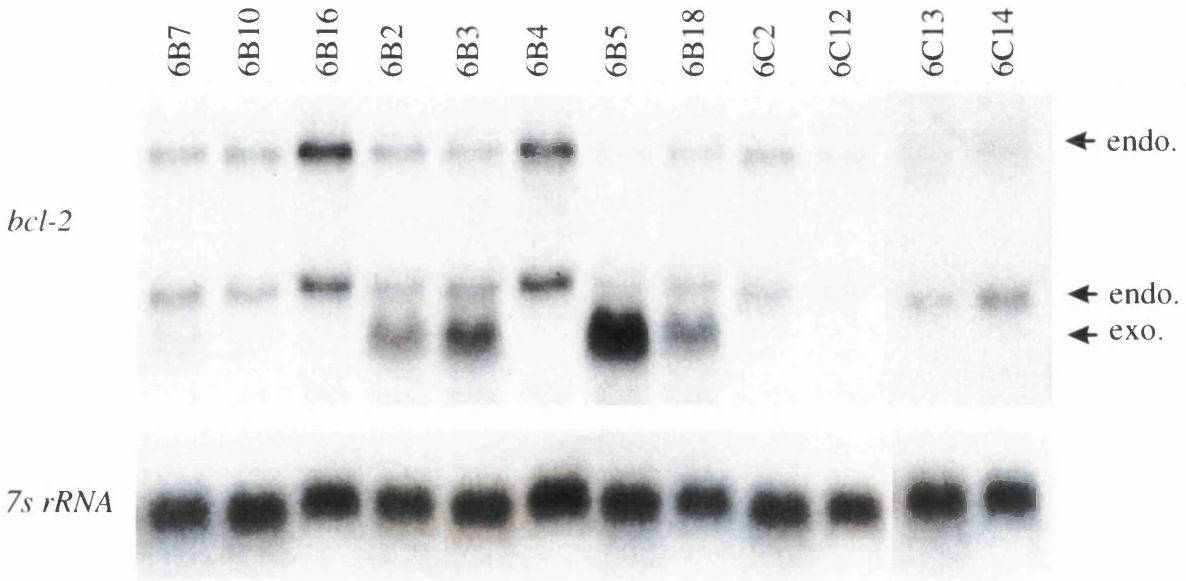
Figure 12.3. Screening of the stably transfected ELM-I/6 clones that express high levels of exogenous *bcl-2* at both mRNA and protein levels by Northern (a) and Western (b) blotting analysis.

The *bcl-2* expression vector, pSSFV(*neo-bcl-2*), was used to generate stably transfected ELM-I/6 clones that expressed high levels of *bcl-2*. The control vector, pSSFV(*neo*), was used in parallel as a control for any artefacts that can be caused by transfection and the subsequent drug selection. The endogenous (endo.) and exogenous (exo.) *bcl-2* transcripts are indicated by arrows in (a). In Western blotting analysis of *Bcl-2* protein expression (b), total protein extracts from a myeloid cell line, M1, was used as a positive control for the *bcl-2* antibody.

Clones transfected with pSSFV(*neo-bcl-2*): 6B2, 6B3, 6B4, 6B5, 6B7, 6B10, 6B16 and 6B18

Clones transfected with pSSFV(*neo*): 6C2, 6C12, 6C13 and 6C14

(a) Northern blotting



(b) Western blotting

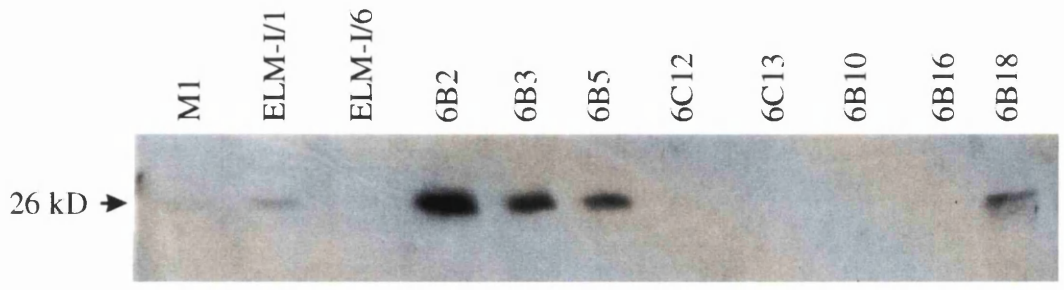
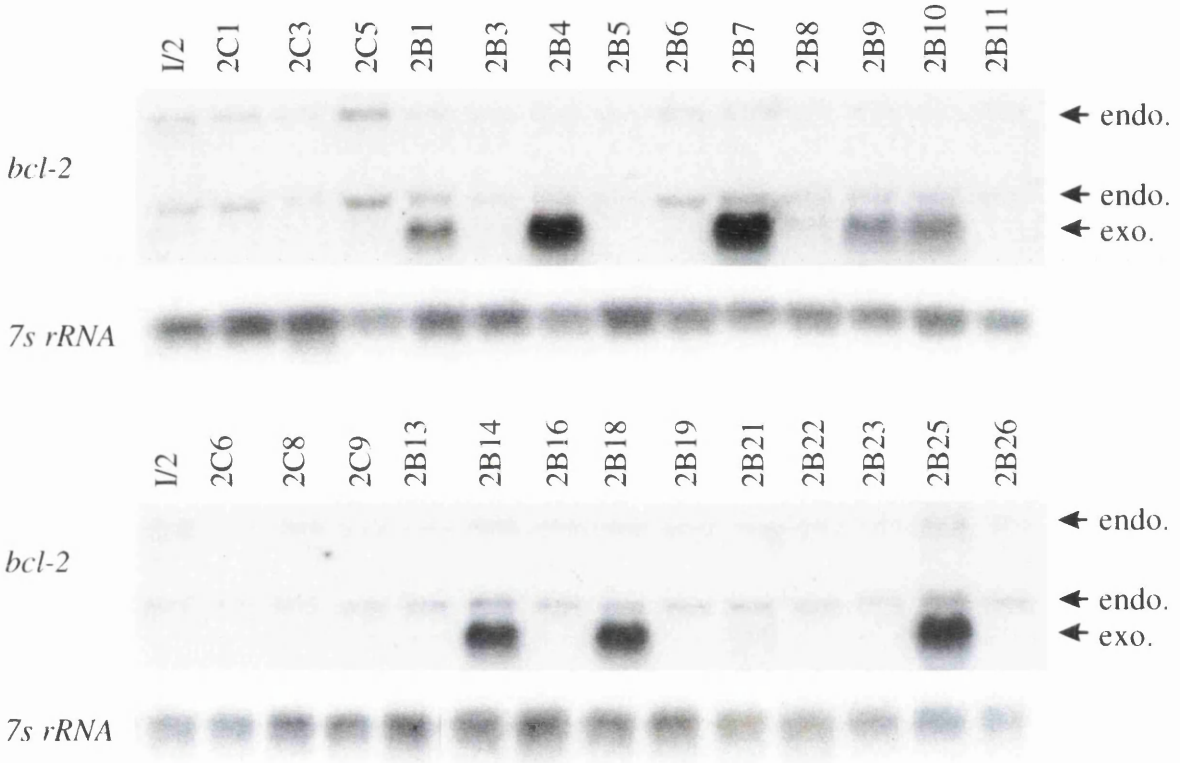


Figure 12.4. Screening of the stably transfected ELM-I/2 clones that express high levels of exogenous *bcl-2* at both mRNA and protein levels by Northern (a) and Western (b) blotting analysis.

The *bcl-2* expression vector, pSSFV(*neo-bcl-2*), was used to generate stably transfected ELM-I/2 clones that expressed high levels of *bcl-2*. The control vector, pSSFV(*neo*), was used in parallel as a control for any artefacts that can be caused by transfection and the subsequent drug selection. The endogenous (endo.) and exogenous (exo.) *bcl-2* transcripts are indicated by arrows in (a).

Clones transfected with pSSFV(*neo-bcl-2*): 2B1, 2B3, 2B4, 2B5, 2B6, 2B7, 2B8, 2B9 2B10, 2B11, 2B13, 2B14, 2B16, 2B18, 2B19, 2B21, 2B22, 2B23, 2B25, and 2B26
Clones transfected with pSSFV(*neo*): 2C1, 2C3, 2C5, 2C6, 2C8 and 2C9

(a) Northern blotting



(b) Western blotting

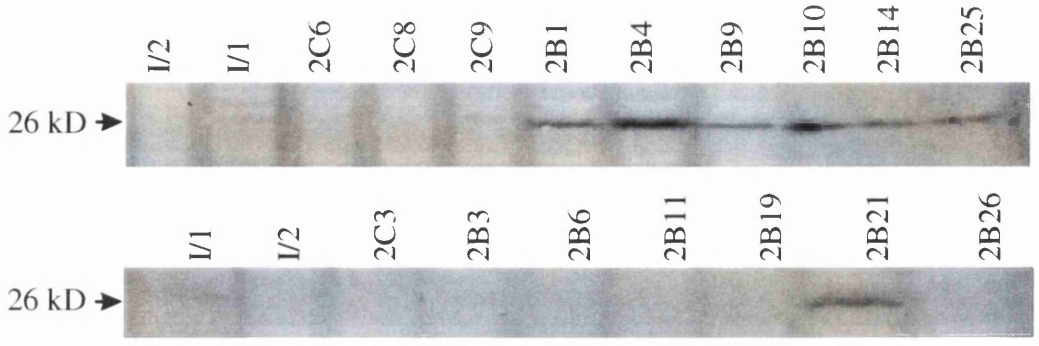
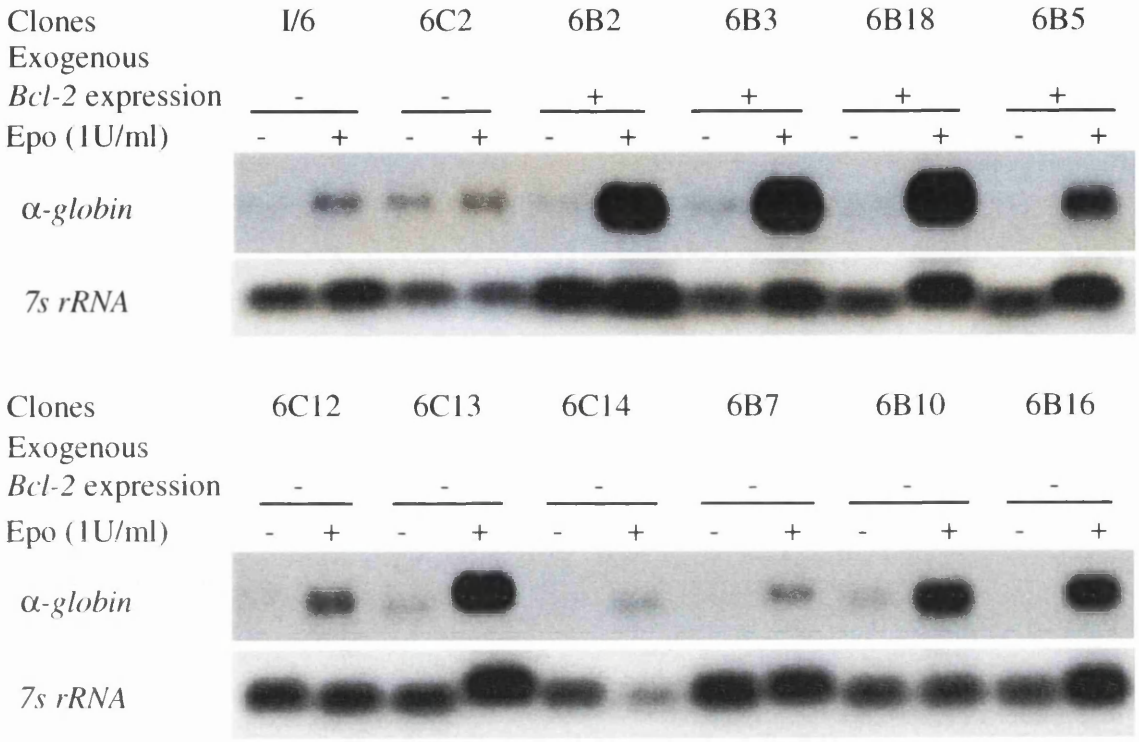
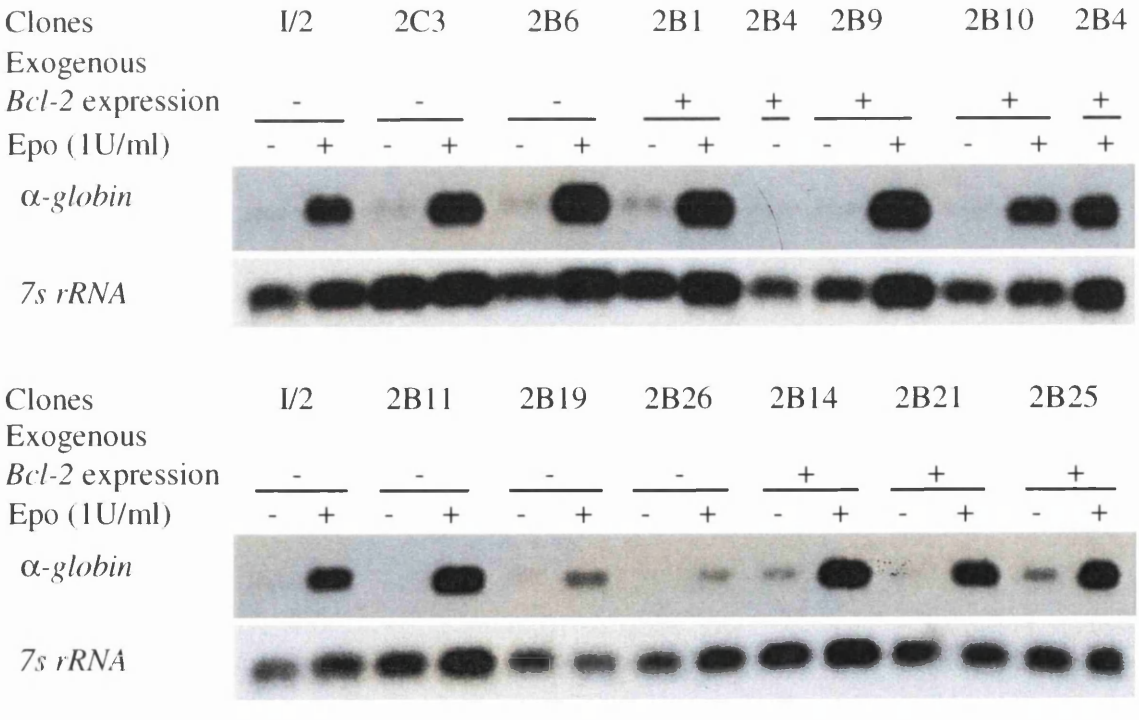


Figure 12.5. Differentiation capability of the stably transfected ELM-I/6 (a) and I/2 (b) clones that constitutively express high levels of exogenous *Bcl-2* protein. The transfectants were cultured in the presence of Epo, harvested at day 3 post-induction, total RNA extracted and analysed by Northern blotting.

(a) ELM-I/6 transfectants



(b) ELM-I/2 transfectants



constitutive up-regulation of *bcl-2* expression is sufficient to induce increased tumorgenicity in ELM cells, ELM-I/2 and I/6 cells that had been stably transfected with the *bcl-2* expression vector and expressed high levels of the *Bcl-2* protein were used in tumorgenicity assays *in vivo*. Clones that had been transfected with the control vector, or do not express exogenous *bcl-2* mRNA were used as controls. Un-transfected ELM-I/1, I/2 and I-6 cells were also used in parallel for comparison. In the *in vivo* tumorgenicity assays, 10^7 viable cells were injected via the tail vein into syngeneic C3H mice that had been immuno-suppressed by irradiation with 300 Rads of γ-rays. The viability of cells injected was checked by the trypan blue exclusion assay (see Methods and Materials) so that the same number of viable cells were used. 4-5 clones were selected from each transfection and separately injected into groups of 9 mice. Mice were sacrificed at 26-30 days post-injection, when their spleens were weighed as a measure of leukaemogenicity. Mice injected with the *bcl-2* transfectants were sacrificed at the same time as the equivalent controls.

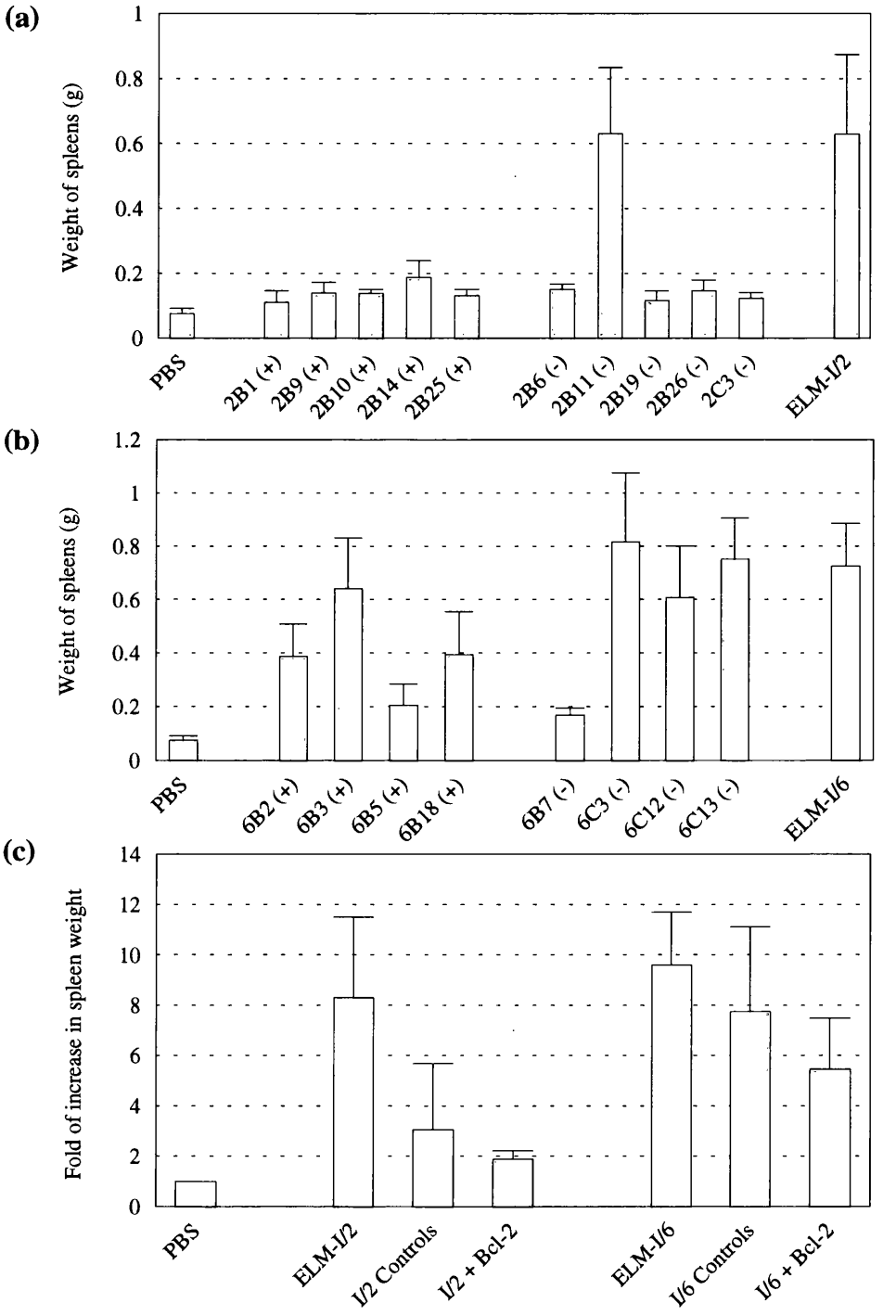
Figure 12.6a and 12.6b show the weight of spleens from mice injected with transfectant clones isolated from ELM-I/2 and I/6 cells, respectively, as a measure of the tumorigenicity of individual clones, and Figure 12.6c shows the average spleen weight as a result of 4-5 clones tested. It can be seen that, although there is variations among the tumorigenicity of individual clones, ectopic expression of high levels of *bcl-2* do not confer increased tumorigenicity *in vivo* in those cells.

12.4. Conclusions

In summary, even though changes in the *bcl-2* expression are associated with erythroid differentiation, they are not absolutely necessary. In addition, constitutive up-regulation of *bcl-2* is not sufficient to induce differentiation arrest or increased tumorigenicity in ELM-I/2 and I/6 cells.

Figure 12.6. Tumorigenicity of the stably transfected ELM-I/2 (a) and I/6 (b) clones that constitutively express high levels of exogenous *Bcl-2* protein.

Tumorigenicity of the stably transfected ELM-I/2 (a) and ELM-I/6 (b) clones was measured by the weights of spleens from mice sacrificed 26-30 days after injection with these cells. Tumorigenicity of un-transfected ELM-I/2 and I/6 cells is also included. Presence (+) or absence (-) of exogenous *bcl-2* expression in each clone is indicated.The average spleen weights as a result of 4-5 clones tested are shown in (c). "I/2 controls" and "I/6 controls" represent ELM-I/2 and ELM-I/6 clones that are either stably transfected with the *bcl-2* expression vector but do not express exogenous *bcl-2*, or stably transfected with the empty vector. "I/2 +*bcl-2*" and "I/6+*bcl-2*" represent ELM-I/2 and I/6 clones stably transfected with *bcl-2* expression vector and express high levels of exogenous *bcl-2* (see Figure 12.3 and 12.4).



PART IV: DISCUSSION AND FUTURE PROSPECTS

CHAPTER 13: Discussion and Future Prospects

13.1. The ELM system as a model for the study of erythropoiesis.

The ELM erythroleukaemia is a very attractive model system to investigate erythropoiesis and erythroleukaemia for the following reasons. First of all, its origin appears to be unique as it was reportedly induced with X-ray irradiation, which usually generates leukaemias derived from other blood cell lineages. Secondly, both the primary tumour and the cell line (ELM-D) subsequently derived are strictly dependent on direct contact with a bone marrow-derived stromal cell layer for long-term growth and survival (Itoh *et al.*, 1988b). Thirdly, ELM-D cells and all the stroma-independent variants apart from one (ELM-I/1) are still able to undergo erythroid differentiation in response to physiological inducers, such as Epo and IL-3. Thus, in contrast to various retrovirally-induced erythroleukaemias, the ELM cells appear to be much less advanced in terms of their loss of normal erythroid progenitor characteristics. Therefore, the ELM system provides a more "normal" model system for studying the stromal control of erythropoiesis. Identifying the growth factors and other regulatory molecules produced by the stroma, and defining their roles in supporting the growth of ELM-D cells, have obvious implications in our understanding of the control of normal and malignant erythropoiesis and the nature of cellular interactions involved. Furthermore, the fact that the various ELM cell lines (ELM-D, I/1, I/2, I/5 and I/6) have different properties in their stroma-dependence, differentiation capacity and tumorigenicity also provides a unique opportunity to identify genetic alterations responsible for generating these different phenotypes (also see Section 13.3). This should give an insight into the nature of intracellular signalling pathways and further our knowledge of the molecular mechanisms of erythropoiesis and leukaemia progression.

By utilising the ELM system, it was demonstrated that a bone marrow-derived stroma pre-adipocyte cell line is able to support the long-term growth and survival of erythroid progenitor cells, and allow erythroid differentiation in response to growth factors. This implies that, although bone marrow is not a major erythropoietic organ in mice, its stromal cells can support erythropoiesis *in vitro* (also see below). This conclusion is consistent with the studies using the Dexter type long-term bone marrow cultures (LTBMCs), which are murine bone marrow primary cultures and contain several cell types including fibroblasts, pre-adipocytes, adipocytes, endothelial cells, osteogenic cells and macrophages (reviewed by Quesenberry, 1992). It has been demonstrated that, apart from supporting differentiation along myeloid pathways, LTBMCs can also support erythropoiesis (Humphries *et al.*, 1979), especially by the addition of Epo (Eliason *et al.*, 1979) or the transfection of the Epo cDNA to the marrow stromal cells

(Corey *et al.*, 1990). Although LTBMCs reconstitute a haemopoietic microenvironment that is a close approximation of the situation *in vivo*, their heterogeneity unavoidably brings about a high level of complexity and has hindered a detailed analysis of the molecular nature of cellular interactions. This has led to the studies, such as ours, which establish bone marrow cell lines from LTBMCs that can support haemopoiesis *in vitro*. However, studies using cloned stromal cell lines have the obvious drawbacks since immortalised or transformed cells are used and, moreover, haemopoiesis is analysed under circumstances where progenitor cells are interacting with only one type of stroma cells. Nevertheless, a more complete picture is likely to emerge when the two strategies are combined.

The significance of the ELM system as a model system for studying the stromal control of erythropoiesis is further reinforced by the research carried out in Dr. M. Obinata's laboratory. They demonstrated that stromal endothelial (Yanai *et al.*, 1989; Yanai *et al.*, 1991) and epithelial-like cell lines (Ohneda *et al.*, 1990) derived from mouse spleen and foetal liver, respectively, are able to support large erythroid colony formation and mimic erythropoiesis *in vivo*. Furthermore, various lines of evidence showed that this kind of *in vitro* microenvironment seems to be created by direct contact and/or short range communication between erythroid progenitor cells and the stromal cell layer (see Section 2.1). This is exactly reminiscent of how the bone marrow-derived stromal cell line supports the long-term growth and survival of ELM-D cells. An intriguing question is whether or not stromal cells in bone marrow, spleen and foetal liver can all support and regulate erythropoiesis *in vivo* and, if so, whether they utilise similar cellular and molecular mechanisms. It is worthwhile noting that cultured stromal cells seem to have lost organ specificity. This is exemplified by the long-term culture for T-cell precursors in that the stromal cell clone used to initiate the cultures could be either bone marrow- or thymus-derived (Zipori and Tamir, 1989). The ability to support precursor T-cell growth is therefore not a property exclusive to the thymic stromal cells. Similarly, the ability to support the stem cell growth and long-term myelopoiesis was exhibited not only by endothelial-adipocytes from mouse bone marrow but also by a clone of thymic stromal cells (Zipori and Tamir, 1989). Two potential explanations may be considered: it is possible that following culture *in vitro*, stromal cells lose their organ specificities; alternatively it is also possible that a variety of stromal cell types exist, each present in all organs but at different frequencies.

Finally, it should be emphasised that any conclusions derived from *in vitro* studies may not be valid when extrapolating to the situations *in vivo*. Therefore, it is very important that studies of the ELM system are complemented with *in vivo* experiments. This can be carried out by the use of congenitally anaemic mutant mouse strains with inherited

environment deficiencies, *in vivo* administration of antibodies and the transgenic approaches.

13.2. Stromal control of the long-term growth of ELM-D cells.

It has been demonstrated in Chapter 9 that, upon stroma-withdrawal, ELM-D cells undergo terminal erythroid differentiation. Moreover, although ELM-D cells grown on the stroma can differentiate in response to Epo, the extent of differentiation is a lot less than that occurs when the stromal cells are removed (see Section 9.5 and Section 12.1.1). This implies that the stromal cells can block differentiation of ELM-D cells, which, to a small extent, can be overridden by Epo, and at least one mechanism whereby the stroma maintains the long-term growth of ELM-D cells is to prevent them from further maturation. Consistent with our finding, Weber and Tykocinski (1994) also demonstrated that a bone marrow-derived fibroblastic cell line can block leukaemic cell differentiation. A role of stroma in inhibiting differentiation may have its physiological significance *in vivo*. It may be a mechanism whereby haemopoietic cells in circulation are tightly controlled so that only cells leaving the bone marrow in response to appropriate cues can survive and proliferate, whereas those getting into circulation "accidentally" due to loss of adhesion to the stroma, would terminally differentiate and die, and therefore would not expand abnormally. On the other hand, those that are induced to differentiate in order to perform certain specific functions may also receive cues to leave the bone marrow and get into circulation where they can differentiate better and are positioned within appropriate tissues.

It would be interesting to identify the molecules involved in differentiation blockage by the MS-5 stromal cells. As discussed in Section 6.2, the stroma-dependence of ELM-D cells strictly requires direct cell-cell contact, suggesting the involvement of cell membrane and adhesion molecules. Indeed, several lines of evidence indicate that the interaction between the transmembrane form of stem cell factor (mSCF) and its receptor, *c-Kit*, is important in maintaining the long-term growth of ELM-D cells. Antibodies against either SCF or *c-Kit* do not affect adhesion between ELM-D and the stroma, whereas they significantly reduce the growth of ELM-D cells on stroma by greater than 60% relative to the untreated controls (J. O'Prey and Prof. W. Ostertag, personal communication). Moreover, mutant Sl/Sl^{d} stromal cells that produce only the soluble form of SCF (Flanagan *et al.*, 1991) are able to support transient but not long-term growth of ELM-D cells (Prof. W. Ostertag, personal communication). The role of SCF/*c-Kit* interaction in inhibiting differentiation is further highlighted by the fact that, in the ELM-I/1 cells which are blocked in erythroid differentiation, the *c-Kit* receptor is constitutively phosphorylated and activated (Dr. N. Leslie, personal communication, also see Section 13.3.4). It would be interesting to see whether over-expression of this

mutated *c-Kit* in ELM-D cells can prevent them from differenting in the absence of stroma and confer stroma-independent growth. However, the fact that all the other stroma-independent variants (ELM-I/2, I/5 and I/6) do not contain constitutively activated *c-Kit* suggests that, even if constitutive activation of *c-Kit* is involved in stroma-independent growth, other mechanism(s) also exist.

The hypothesis that the stromal cells maintain the long-term growth of ELM-D cells by preventing them from further maturation does not exclude the possibility that the stromal cells also provide survival and proliferation signals. Indeed, a block in differentiation in ELM-D cells in the absence of stroma may not be sufficient for their survival and proliferation. It is likely that a combination of mechanisms are at play in coffering stroma-independent growth.

Other molecules that are also involved in the maintenance of long-term growth of ELM-D cells by the stroma are insulin growth factor-1 (IGF-1) and integrin α1. Neutralising antibody against IGF-1 reduces the long-term growth of ELM-D cells on the stroma by 50%, and so does blocking antibody against integrin α1, although to a less degree (J. O'Prey, personal communication). Furthermore, when both antibodies against *c-Kit* and IGF-1 are applied, the growth of ELM-D cells on the stroma is completely abolished, suggesting that interactions of SCF and IGF-1 with their respective receptors contribute to the majority of the stromal cell activity in maintaining the long-term growth of ELM-D cells.

13.3. Genetic characterisation and functional analysis of the ELM system.

As mentioned above, the various ELM cell lines differ in stroma-dependence, differentiation potential and tumorigenicity and thus provide us a unique opportunity to identify genetic events involved in generating these different phenotypes. Indeed, it has been demonstrated that different ELM cell lines have different gene expression profiles (see Chapter 10).

13.3.1. *fli-1* and *bcl-2*.

Most significantly, it was found that expression of *bcl-2* and *fli-1* at both mRNA and protein levels is largely elevated in one of the stroma-independent variants, ELM-I/1, which is the only clone that is blocked in erythroid differentiation in response to various inducers and also displays the highest tumorigenicity. When the changes in *bcl-2* expression in differentiating ELM cells were studied (see Section 12.1), it was found that, in ELM-D cells undergoing Epo-induced erythroid differentiation in the presence of stromal cells, the expression of *bcl-2* mRNA is stably decreased. Furthermore, in

response to Epo, the differentiation of ELM-I/2 and I/6 cells is also accompanied by an early decline in the *bcl-2* mRNA expression, which is followed by a later increase. In contrast, the expression of *bcl-2* mRNA in ELM-I/1 cells is not affected by Epo. This, together with the quantitative changes of *bcl-2* expression during cell differentiation observed in other laboratories (Hockenbery *et al.*, 1991; Gratiot-Deans *et al.*, 1993; Merino *et al.*, 1994; Marthinuss *et al.*, 1995; Zhang *et al.*, 1996), prompted us to address a role of *Bcl-2* in erythroid differentiation. It was proposed that a decline in the *bcl-2* expression is necessary for ELM cells to undergo erythroid differentiation, and therefore in ELM-I/1 cells, where the *bcl-2* expression is constitutively elevated, the differentiation program is blocked. Then the question is: is *bcl-2* up-regulation the only factor responsible for the differentiation arrest in ELM-I/1 cells? In another words, is *bcl-2* up-regulation sufficient to induce differentiation arrest in ELM-I cells? In order to test this, a *bcl-2* expression vector was transfected into ELM-I/2 and I/6 cells, and stably transfected clones selected. However, clones that ecotopically express high levels of *bcl-2* are still able to differentiate in response to Epo (see Section 12.2), and do not display significantly elevated tumorigenicity *in vivo* (see Section 12.3). This implies that either *Bcl-2* is not involved in inducing differentiation arrest and increased tumorigenicity, or that over-expression of *bcl-2* on its own is not sufficient. The latter possibility may exemplify the requirement for oncogene co-operation in tumorigenesis. Indeed, *bcl-2* was one of the first oncogenes where this requirement was documented on the basis of the fact that cross-breeding *bcl-2* and *c-myc* transgenic mice induces a rapidly emerging undifferentiated haemopoietic leukaemia (Strasser *et al.*, 1990).

The possibility that *Bcl-2* requires other co-factors to generate the full phenotype in ELM-I/1 cells is further supported by the fact that *fli-1*, like *bcl-2*, is over-expressed in ELM-I/1 cells, and the finding that the *Fli-1* protein can transactivate the *bcl-2* promoter and may well be directly responsible for the increased expression of *bcl-2* in ELM-I/1 cells. As a member of a family of multi-functional transcription factors, it is most likely that *Fli-1* has a number of target genes and its activation in ELM-I/1 cells may alter the expression of several other genes apart from *bcl-2*. It is anticipated that, to achieve the full phenotype of the ELM-I/1 variant, multiple genetic changes are required. It would be interesting to see if over-expression of *fli-1* in ELM-I/2, 5 and 6 cells can induce differentiation arrest and increased tumorigenicity, which is an ongoing project being carried out by Dr. C. Bartholomew in our laboratory. Conversely, it would also be worthwhile to perform expression- or functional "knockout" experiments of *bcl-2* or *fli-1* in ELM-I/1 cells, and see whether or not the phenotypes are somewhat reversed. This can be carried out either by an antisense approach or, in the case of *fli-1*, by over-expressing the cDNA of a *fli-1* dominant negative mutant which consists of the DNA-binding domain only and can act as a competitive inhibitor of the normal protein (see Section 11.1.1). This kind of "knockout" approaches can be more informative because if

several factors are necessary to generate a phenotype then eliminating any one of them at a time is likely to see a partial reversion.

In search of other factors that may be responsible for the differentiation arrest and increased tumorigenicity in ELM-I/1 cells, *c-myc* and *c-myb* are of particular interest. *C-myc* and *c-myb* are proto-oncogenes that play an important role in regulating cell proliferation, differentiation and apoptosis. As mentioned above, *bcl-2* and *c-myc* display potent synergy in tumour development in transgenic mice. Furthermore, it has been well documented that the expression of *c-myc* and *c-myb* mRNA shows a rapid biphasic change in murine erythroleukaemia cells (MEL) induced to differentiate, and constitutive expression of either of them results in differentiation arrest (Dmitrovsky *et al.*, 1986; Clarke *et al.*, 1988). Moreover, the *Ets* proteins have been implicated in transcriptional regulation of the *c-myc* expression (Roussel *et al.*, 1994). In addition, *c-myb* can co-operate with the *Ets* proteins in transcriptional activation (see Section 4.1.2). It has been reported that *c-Ets-2* does not activate the *mim-1* promoter unless *c-Myb* is also present (Dudek *et al.*, 1992). Furthermore, *c-Myb* can synergise with either *c-Ets-1* or *c-Ets-2* to transactivate the promoter of an early myeloid gene (Shapiro, 1995). This co-operation between *c-Myb* and the *Ets* proteins is of particular interest in light of the fact that *v-Myb* can also transactivate the *bcl-2* promoter, and over-expression of *v-Myb* leads to an up-regulation of the *bcl-2* mRNA (Frampton *et al.*, 1996). It would be interesting to see whether *c-Myb* and *Fli-1* co-operate in up-regulating the *bcl-2* expression in ELM-I/1 cells.

13.3.2. *erg.*

As mentioned in Section 10.1, the stroma-independent variants (ELM-I/1, I/2, I/5 and I/6) express an increased level of *erg* mRNA in comparison to their stroma-dependent parent (ELM-D) (also see Nibbs *et al.*, 1993). Furthermore, it has also been reported by Nibbs *et al.* (1993) that, upon stroma-withdrawal, the expression of *erg* mRNA in ELM-D cells is elevated. Therefore , it was postulated that *erg* up-regulation is associated with the stroma-independent growth and this is made permanent in ELM-I cells. However, preliminary studies of the expression of *Erg* protein using an anti-human *Erg* antibody demonstrated that the *Erg* protein is only readily detectable in ELM-I/1 cells. Based on this finding, the significance of the different levels of *erg* mRNA expression between ELM-D and ELM-I cells, and the up-regulation of *erg* mRNA in ELM-D cells upon stroma-withdrawal, becomes uncertain. Since it is very important to verify the expression of *Erg* protein in ELM cells, it may be worthwhile to check the expression of *Erg* protein using another *Erg* antibody. If our finding is confirmed, then it would be interesting to see whether, like *Fli-1*, *Erg* can up-regulate *bcl-2*, or whether *Erg* is responsible for the differentiation arrest and increased tumorigenicity in ELM-I/1 cells.

On the other hand, if the expression of *erg* at the protein level is consistent with that at the mRNA level, then functional studies are desired to address a role of *Erg* in confering stroma-independent growth. This can be carried out by over-expressing *erg* in ELM-D cells to see if this can induce stroma-independent growth. Alternatively, an antisense approach or the approach involving dominant negative mutants of *erg* can be applied in ELM-I cells to see whether "knocking out" *erg* expression or functions can reverse these cells to become stroma-dependent.

13.3.3. *gata-1* and *scl/tal-1.*

In addition to *fli-1* and *bcl-2* activation, a number of other genetic changes has also been identified in ELM-I/1 cells. Two transcription factors that are positive regulators of erythropoiesis, *gata-1*and *scl/tal-1* (hereafter called *scl*), are expressed at an elevated level according to Northern blotting analysis (see Section 10.1). In order to pursue this further, it would first be essential to see if the expression of *GATA-1* and *SCL* proteins is also significantly increased. If this is the case, it would appear paradoxical since ELM-I/1 cells are blocked in erythroid differentiation and the logical expectation would be that they lack certain positive regulators. However, it has been documented that ecotopic expression of *scl* in late myeloid progenitors and early leukaemic precursors induces a proliferative stimulus under sub-optimal culture conditions via an anti-apoptotic effect or stimulation of DNA synthesis, and these effects are accompanied by a marked inhibitory effect on differentiation (Condorelli *et al.*, 1997). It is well known that the specific effect of a multi-functional protein such as *SCL* is largely dependent upon a particular cellular context. Therefore, whether *SCL* promotes or inhibits erythroid differentiation in the ELM cells may also depend on other factors present. Moreover, the potential role of *SCL* in promoting proliferation may also underlie the molecular mechanisms responsible for the increased tumorigenicity associated with this particular cell line.

In addition, it is also possible that ELM-I/1 cells may express mutant forms of *gata-1* or *scl*. The mutation could result in the expression of aberrant proteins that are defective in DNA-binding or transcriptional activity. Alternatively, the mutation could also cause a defect in post-translational regulation of the protein, in particular, by phosphorylation in response to differentiation inducers. It has been reported that both *GATA-1* and *SCL* can be phosphorylated by differentiation inducers, such as DMSO and Epo (Crossley and Orkin, 1994; Prasad *et al.*, 1995). Such induced phosphorylation may trigger functional activation of the proteins as transcription factors and the subsequent involvement in erythroid differentiation. Finally , it is also important to determine the expression in ELM-I/1 cells of any of the co-factors that are known to interact with *GATA-1* or *SCL* and work in conjunction to regulate erythropoiesis. It has been mentioned in Section 2.2

that direct protein-protein interactions largely dictate the functional activity of *GATA-1* and *SCL*. In the case of *SCL*, it would be interesting to see if *LMO-2* and *Id* proteins are involved in the regulation of erythroid differentiation of ELM cells.

13.3.4. *c-kit.*

In parallel to the genetic characterisation work presented in this thesis, Dr. N. Leslie in our laboratory has also identified mutations in *c-kit*, which encodes the receptor for stem cell factor (SCF), in ELM-I/1 cells, but not in any other of the ELM cell lines. Although *c-kit* is expressed at a similar level in all the ELM cells tested (ELM-D, I/1, I/2, I/5 and I/6), only the *c-Kit* protein in ELM-I/1 exhibits constitutive phosphorylation in the absence of growth stimuli. Under normal circumstances, phosphorylation of the *c-Kit* protein is induced by ligand-binding by SCF, which results in activation of the protein and subsequently initiates a series of signalling pathways leading towards promoting cell survival and proliferation. In light of that, it would be interesting to see whether certain target molecules downstream of *c-Kit* are also constitutively activated. It is anticipated that, as a result of constitutive activation of *c-Kit*, ELM-I/1 cells may reside in a hyper-proliferative status, which may be obstacle for differentiation. In order to test this, the mutant *c-kit* derived from ELM-I/1 cells has been over-expressed in other ELM-I cells to see whether or not this will induce differentiation arrest and increased tumorigenicity. This would be interesting in light of the fact that activation of the *c-Kit* signalling pathway has been implied as a necessary event in erythroleukaemia induction (Ben-David and Bernstein, 1991; also see Section 3.2.3).

13.4. Molecular mechanisms of transactivation of the *bcl-2* promoter by *Fli-1*.

As mentioned above, ELM-I/1 cells display significantly elevated expression of *fli-1* and *bcl-2* at both mRNA and protein levels. This, together with the fact that an important regulatory element (NRE) in the *bcl-2* promoter contains multiple potential *Ets*-binding sites (EBS) (see Figure 5.1), suggested that it might be worthwhile to determine whether *Fli-1* can transactivate the *bcl-2* promoter and is responsible for the up-regulation of *bcl-2* expression in this particular cell line. As demonstrated in Chapter 11, *Fli-1*, but not its truncated mutant (DMN2), can transactivate a partial *bcl-2* promoter in a quail fibroblast cell line, Q2BN. Moreover, when the *bcl-2* promoter reporter construct was transfected into ELM-I/1 and I/2 cells, it displayed higher activity in the former, in which endogenous *fli-1* expression is significantly activated, than in the latter, which do not express *fli-1* at all. DNase I footprinting analysis of a minimum *bcl-2* promoter fragment has identified six footprints, four of which (FP2, 3, 4 and 6) contain potential EBS, and FP6 shows different footprinting pattern between the ELM-I/1 and I/2 nuclear extracts. When oligonucleotides from the footprinted regions were analysed in gel

retardation assays, it was found that FP3 and FP6 show differential binding to the ELM-I/1 nuclear extracts compared to the ELM-I/2 extracts. Although all the FP oligonucleotides seem to bind to the extracts specifically as judged by self-competition experiments, none of them can be competed significantly with oligonucleotides containing binding sites for known *Ets* proteins, including E74, Ets-3, Fli, PEA3 and PU.1. Finally, "supershift" analysis using *Fli-1*-specific antibody was not able to reveal the binding between *Fli-1* and any of the six FPs.

There are several possibilities why gel retardation and "supershift" analysis did not reveal a direct interaction between *Fli-1* and the six FP oligonucleotides. First, it is possible that *Fli-1* can interact with the FP oligonucleotides, and this interaction results in a conformational change in the protein that masks the epitope which would normally bind to the *Fli-1* antibody. To test this possibility, a panel of *Fli-1*-specific antibodies can be raised against various regions of the protein, and see whether any of them would have an effect on *Fli-1*-binding to the six FP oligonucleotides.

Secondly, it is possible that the *Fli-1* antibody can recognise and bind to *Fli-1* in a protein:DNA complex, but this interaction does not survive in the gel electrophoresis conditions used and the antibody is thus released, whereas *Fli-1* remains in the complex. This possibility can be tested by using the biotinylated oligonucleotide capture approach developed by Dr. B. Ozanne in the institute, in which oligonucleotides are contemerised and bound to beads which are then incubated with nuclear extracts. Following centrifugation, the unbound fraction is removed, the protein:DNA complex recovered from the beads and subject to Western blotting analysis.

A third possibility is that binding of *Fli-1* requires flanking sequences outside the actual footprinted regions. It should be born in mind that that DNA secondary structure is important not only for protein recognition and binding but also for the stabilisation of the protein:DNA complex. Analysing short stretches of DNA sequence unavoidably destroys this structure. This hypothesis is supported by the evidence that adjacent FPs tend to compete with one another for protein binding in gel retardation assays (see Figure 11.14 and Table 11.1), which implies that some protein factors in the nuclear extracts can bind to several regions within the promoter, and it is likely that a "multi-protein complex bridge" is built across these regions stablised by DNA secondary structure. Therefore, it is worthwhile to use longer oligonucleotides in gel retardation and "supershift" analysis, which contain two or more footprints.

However, it may also be actually true that *Fli-1* does not transactivate the bcl-2 promoter by directly binding to the DNA. The fact that neither E74 or Fli oligonucleotides competes with any of the FPs oligonucleotides, although they do bind

to both *in vitro* translated *Fli-1* and the *Fli-1* protein present in the ELM-I/1 nuclear extracts, suggests that this might be the case. Instead, *Fli-1* may bind to other protein factor(s) in a protein:DNA complex. Such an interaction between *Fli-1* and another factor might not be resolved in gel retardation and "supershift" assays. There are several precedents for such interactions: since transactivation by many *Ets* proteins has been shown to require multiple co-factors that physically interact with them (see Section 4.1.2). In particular, *Elk-1* (Hipskind *et al.*, 1991), *SAP1* (Dalton and Treisman, 1992) and *Fli-1* (Watson *et al.*, 1997) can recognise and interact with the serum response factor (SRF) bound to the DNA, and form a stable ternary complex, but not the naked DNA. To determine whether *Fli-1* was present in those multiple protein:DNA complexes responsible for the footprints observed in DNase I footprinting analysis, it would be worthwhile to perform immuno-depletion prior to footprinting assays and see if footprinting patterns are altered. However, it should bear in mind that since many protein factors can be involved in binding to a promoter region, the changes due to the lack of one protein may be subtle. To identify those unknown factor(s) that bind to the minimum *bcl-2* promoter and *Fli-1*, and further dissect the molecular mechanisms whereby *Fli-1* transactivates the *bcl-2* promoter, the yeast one and two hybrid systems can be used.

The fact that the molecular mechanism(s) whereby *Fli-1* transactivates the *bcl-2* promoter is still elusive does not exclude a regulatory role of *Fli-1* in regulating the *bcl-2* expression. This is emphasised by the discovery that the *fli-1* and *bcl-2* transgenic mice display certain similar phenotypes (see Section 4.2). These similarities indicate that these two proteins may have similar functional end points. One possibility, tentatively suggested by the present finding, is that *Fli-1* and *Bcl-2* are part of the same pathway and, in this scenario, it is possible that *Fli-1* may lie upstream of *Bcl-2* and regulate its activity, directly or indirectly. However, it is also possible that they act through distinct pathways which converge at some point.

PART V: REFERENCES

D'Andrea, A.D. (1994). Hematopoietic growth factors and the regulation of differentiative decisions. Curr. Opin. Cell Biol. 6, 804-808.

Aplan, P.D., Lombardi, D.P., Ginsberg, A.M., Cossman, J., Bertness, V. and Kirsch, I.R. (1990). Disruption of the human scl locus by illegitimate V-(D)-J recombinase activity. Science 250, 1426-1429.

Aplan, P.D., Nakahara, K., Orkin, S.H. and Kirsch, I.R. (1992). The SCL gene product: a positive regulator of erythroid differentiation. EMBO J. 11, 4073-4-81.

Athanosiou, M., Clausen, P.A., Mavarothalassitis, G.J., Zhang, X.K., Watson, D.K. and Blair, D.G. (1996). Increased expression of the Ets-related transcription factor Fli-1/ErgB correlates with and can induce the megakaryocytic phenotype. Cell Growth & Differentiation 7, 1525-1534.

Baron, M.H. and Farrington, S.M. (1994). Positive regulators of the lineage-specific transcription factor GATA-1 in differentiating erythroid cells. Mol. Cell. Biol. 14, 3108-3114.

Bassuk, A.G. and Leiden, J.M. (1995). A direct physical association between Ets and AP-1 transcription factors in normal human T-cells. Immunity 3, 223-227.

Ben-David, Y. and Bernstein, A. (1991). Friend virus-induced erythroleukaemia and the multistage nature of cancer. Cell 66, 831-834.

Ben-David, Y., Giddens, E.B., Letwin, K., Bernstein, A. (1991). Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. Genes & Develop. 5, 908-918.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L. and Weintraub, H. (1990). Cell 61, 40-59.

Bernstein, A., Forrester, L., Reith, A., Dubreuil, P. and Rottapel, R. (1991). The murine w/c-kit and steel loci and the control of hematopoiesis. Semin. Hematol. 28, 138-142.

Beug, H., Mullner, E.W. and Hayman, M.J. (1994). Insights into erythroid differentiation obtained from studies on avian erythroblastosis virus. Curr. Opion. Cell Biol 6, 816-824.

Bhat, N.K., Thompson, C.B., Lindsten, T., June,C.H., Fujiwara, S., Koizumi, S., Fisher, R.J. and Papas, T.S. (1990). Reciprocal expression of human ets1 and ets2 genes during T-cell activation - regulatory role for the proto-oncogene ets1. Proc. Natl. Acad. Sci. USA 87, 3723-3727.

Bissonnette, R.P., Echeverri, F., Mahboubi, A. and Green, D.R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature 359, 552-554.

Blobel, G.A., Sieff, C.A. and Orkin, S.H. (1995). Ligand-dependent repression of the erythroid transcription factor GATA-1 by the estrogen receptor. Mol. Cell. Biol. 15, 3147-3153.

Blobel, G.A. and Orkin, S.H. (1996). Estrogen induced apoptosis by inhibition of the erythroid transcription factor GATA-1. Mol. Cell. Biol. 16, 1687-1694.

Bockamp, E.O., McLaughlin, F., Murrell, A. and Green, A.R. (1994). Transcription factors and the regulation of haemopoiesis: lessons from GATA and SCL proteins. BioEssays 16, 481-488.

Bories, J.-C., Willerford, D.W., Grevin, D., Davidson, L., Camus, A., Martin, P., Stehelin, D. and Alt, F.W. (1995). Increased T-cell apoptosis and terminal B-cell differentiation induced by inactivation of the Ets-1 proto-oncogene. Nature 377, 635-638.

Bosselut, R., Levin, J., Adjady, E. and Ghysdael, J. (1993). A single amino-acid substitution in the Ets domain laters core DNA binding specificity of Ets1 to that of the related transcription factores Elf1 and E74. Nuclei Acid Res. 21, 5184-5191.

Bradbury, D., Zhu, Y.M. and Russell, N. (1994). Regulation of bcl-2 expression and apoptosis in acute myeloblastic leukaemic cells by granulocyte-macrophage colony-stimulating factor. Leukemia 8, 786-791.

Briegel, K., Bartunek, P., Stengl, G., Lim, K.C., Beug, H., Engel, J.D. and Zenke, M. (1996). Regulation and function of transcription factor GATA-1 during red blood cell differentation. Development 122, 3839-3850.

Buttice, G. and Kurkinen, M. (1993). A polyoma virus enhancer A-binding protein-3 site and Ets-2 protein have a major role in the 12-*O*-tetradecanoylphorbol-13-acetate response of the human stromelysin gene. J. Biol. Chem. 268, 7196-7204.

Buttice, G., Duterque-Coquillaud, M., Basuyaux, J.P., Carrere, S., Kurkinen, M. and Stehelin, D. (1996). Erg, an Ets-family member, differentially regulates human collagenase 1 (MMP1) and stromelysin 1 (MMP3) gene expression by physically interacting with the Fos/Jun complex. Oncogene 13, 2297-2306.

Carroll, M., Tomasson, M.H., Barker, G.F., Golub, T.R. and Gilliand, D.G. (1996). The Tel platelet-derived growth factor-β receptor (PDGF-β-R) fusion in dhronic myelomonocytic leukaemia is a transforming protein that self-associates and activates PDGF-β-R kinase-dependent signalling pathways. Proc. Nat. Acad. Sci. USA 93, 14845-14850.

Chen, Q., Cheng, J.T., Tsai, L.H., Schneider, N., Buchanan, G., Carroll, A., Crist, W., Ozanne, B., Siciliano, M.J. and Baer, R. (1990). The tal gene undergoes chromosomal translocation in T cell leukaemia and potentially encodes a helix-loop-helix protein. EMBO J. 9, 415-424.

Chowdhury, K. (1991). One step 'miniprep' method for the isolation of plasmid DNA. Nucleic Acids Res. Vol. 19(10): 2792.

Clarke, M.F., Kukowskalatallo, J.F., Westin, E., Smith, M. and Prochownik, E.V.(1988). Constitutive expression of a c-myb cDNA block Friend murine erythroleukaemia cell differentiation. Mol. Cell. Biol. 8, 884-892.

Condorelli, G., Vitelli, L., Valtieri, M., Marta, I., Montesoro, E., Lulli, V., Baer, R. and Peschle, A. (1995). Coordinate expression and developmental role of Id2 protein and TAL1/E2A heterodimer in erythroid progenitor differentiation. Blood 86, 164-175.

Condorelli, G.L., Tocci, A., Botta, R., Facchiano, F., Testa, U., Vitelli, L., Valtieri, M., Croce, C.M. and Peschle, C. (1997). Ectopic TAL-1/SCL expression in phenotypically normal or leukemic myeloid precursors: proliferative and antiapoptotic effects coupled with a differentiation blockade. Mol. Cell. Biol. 17, 2954-2969.

Corey, C.A., Desilva, A. and Williams, D.A. (1990). Erythropoiesis in murine long-term marrow cultures following transfer of the erythropoietin cDNA into marrow stromal cells. Exp. Hematol. 18, 201-204.

Crepieux, P., Coll, J. and Stehelin, D. (1994). The Ets family of proteins: weak modulators of gene expression in quest for transcriptional partners. Crit. Rev. Oncogenesis 5, 615-638.

Cross, M.A., Heyworth, C.M., Murrell, A.M., Bockamp, E.O., and Dexter, T.M. and Green, A.R. (1994). Expression of lineage restricted transcription factors precedes lineage-specific differentiation in a multipotent hematopoietic progenitor-cell line. Oncogene 9, 3013-3016.

Crossley, M. and Orkin, S.H. (1994). Phosporylation of the erythroid transcription factor GATA-1. J. Biol. Chem 269, 16589-16596.

Dai, C.H., Krantz, S.B. and Zsebo, K.M. (1991). Human burst-forming units-erythroid need direct interaction with stem cell factor for further development. Blood 78, 2493-2497.

Dalton, S. and Treisman, R. (1992). Characterisation of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell 68, 597-612.

Delattre, O., Zucman, J., Plougastel, B., Desmaze, C., Melot, T., Peter, M., Kovar, H., Joubert, I., de Jong, P., Rouleau, G., Aurias, A. and Thomas, G. (1992). Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature 359, 162-165.

Delgado, M.D., Hallier, M., Meneceur, P., Tavitian, A. andMoreaugachelin, F. (1994). Inhibition of Friend-cells proliferation by Spi-1 antisense oligodeoxynucleotides. Oncogene 9, 1723-1727.

Deveaux, S., Filipe, A., Lemarchandl, V., Ghysdael, J., Romeo, P.H. and Mignotte, V. (1996). Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. Blood 87, 4678-4685.

Dmitrovsky, E., Kuehl, W.M., Hollis, G.F., Kirsch, I.R., Bender, T.P. and Segal, S. (1986). Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukaemia cell line. Nature 322, 748-750.

Donaldson, L.W., Petersen, J.M., Graves, B.J. and McIntosh, L.P. (1996). Solution structure of the ETS domain from murine Ets-1: a winged helix-turn-helix DNA binding motif. EMBO J. 15, 125-134.

Doyle, K., Zhang, Y., Baer, R. and Bina, M. (1994). Distinguishable patterns of protein-DNA interactions involving complexes of basic helix-loop-helix proteins. J. Biol. Chem. 269, 12099-12105.

Dudek, H., Tantravahi, R.V., Rao, V.N., Reddy, E.S.P. and Reddy, E.P. (1992). Myb and Ets proteins cooperate in transcriptional activation of the min-1 promoter. Proc. Natl. Acad. Sci. USA 89, 1291-1295.

Duke, R.C. and Cohen, J.J. (1986). IL-2 addiction - withdrawal of growth factor activates a suicide program in dependent T-cells. Lymphokine Res. 5, 289-299.

Duterque-Coquillaud, M., Niel, C., Plaza, S. and Stehelin, D.(993). New human erg isoforms generated by alternative splicing are transcriptional activors. Oncogene 8, 1865-1873.

Elefanty, A.G., Antoniou, M., Custodio, N., Carmo-Fonseca, M. and Grosveld, F.G. (1996). GATA transcription factors associate with a novel class of nuclear bodies in erythroblasts and megakaryocytes. EMBO J. 15, 319-333.

Eliason, J.F., Testa, N. and Dexter, T.M. (1979). Erythropoietin-stimulated erythropoiesis in long-term marrow culture. Nature 281, 382-384.

Fanidi, A., Harrington, E.A. and Evan, G.I. (1992). Cooperative interaction between c-myc and bcl-2 protooncogenes. Nature 359, 554-556.

Farrow, S.N. and Brown, R. (1996). New members of the Bcl-2 family and their protein partners. Curr. Opinion Gen. & Devel. 6: 45-49.

Fernandez-Sarabia, M.J. and Bischoff, J.R. (1993). Bcl-2 accociates with the ras-related protein R-ras p23. Nature 366, 274-275.

Finger, L.R., Kagan, J., Christopher, G., Kurtzberg, J., Hershfield, M.S., Nowell, P.C. and Croce, C.M. (1989). Involvement of the TCL5 gene on human chromosome-1 in T-cell leukemia and melanoma. Proc. Natl. Acad. Sci. USA 86, 5039-5043.

Flanagan, J.G., Chan, D.C. and Leder, P. (1991). Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. Cell 64, 1025-1035.

Frampton, J., Ramqvist, T. and Graf, T. (1996). v-Myb of E26 leukemia viurs up-regulates bcl-2 and suppresses apoptosis in myeloid cells. Genes & Devel. 10, 2720-2731.

Fraser, M.J. (1994). Endo-exonucleases: enzymes involved in DNA repair and cell death? BioEssays 16, 761-766.

Fujiwara, S., Fisher, R.J., Bhat, N.K., Diaz de la Espina, S.M. and Papas, T.S. (1988). A short-lived nuclear phosphoprotein encoded by the human ets-2 proto-oncogene is stabilised by activation of protein kinase C. Mol. Cell. Biol. 8, 4700-4706.

Gibson, L.F., Piktel, D., Narayanan, R., Nunez, G. and Landreth, K.S. (1996). Stromal cells regulate bcl-2 and bax expression in pro-B cells. Exp. Hemat. 24, 628-637.

Gilles, F., Raes, M.B., Stehelin,m D., Vandenbunder, B. and Fafeur, V. (1996). The c-ets-1 protooncogene is a new early-response gene differentially regulated by cytokines and growth factors in human fibroblasts. Exp. Cell Res. 222, 370-378.

Golub, T.R., Barker, G.F., Lovett, M. and Gilliland, D.G. (1994). Fusion of PDGF receptor β to a novel ets-like gene, tel, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. Cell 77, 307-316.

Green, A.R., Lints, T., Visvader, J., Harvey, R. and Begley, C.G. (1992). SCL is coexpressed with GATA-1 in hemopoietic cells but is also expressed in developing brain. Oncogene 7, 653-660.

Green, A.R., Rockman, S., De Luca, E. and Begley, C.G. (1993). Induced myeloid differentiation of K562 cells with down-regulation of erythroid and megakaryocytic transcription factors - a novel experimental-model for hematopoietic lineage restriction. Exp. Haematol. 21, 525-531.

Green, T. (1996). Haematopoiesis: master regulator unmasked. Nature 383, 575.

Hagemeier, C., Bannister, A.J., Cook, A. and Kouzarides, T. (1993). The activation domain of the transcription factor PU.1 binds the Rb protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. Proc. Natl. Acad. Sci. USA 90, 1580-1584.

Haldar, S., Jena, N. and Croce, C.M. (1995). Inactivation of Bcl-2 by phosphorylation. Proc. Natl. Acad. Sci. USA 92, 4507-4511.

Haldar, S., Basu, A. and Croce, C.M. (1997). Bcl-2 is the guardian of microtubule integrity. Cancer Res. 57, 229-233.

Hamamura, K., Matsuda, H., Takeuchi, Y., Habu, S., Yagita, H. and Okumura, K. (1996). A critical role of VLA-4 in erythropoiesis in vivo. Blood 87, 2513-2517.

Hankins, W.D., Kost, T.A., Koury, M.J. and Kranz, S.B. (1978). Erythroid bursts produced by Friend leukemia virus in vitro. Nature 276, 506-508.

Hankins, W.D., Chin, K.L., Dons, R. and Signounas, G. (1989). Erythropoietin-dependent and erythropoietin-producing cell lines - implications for research and for leukemia therapy. Ann. NY Acad. Sci. 554, 21-28.

Harrison, P.R., Conkie, D. and Paul, J. (1973). Role of cell division and nucleic acid synthesis in erythropoietin-induced maturation of foetal liver cells in vitro. In *British Society for Developmental Biology Synposium: The Cell Cycle in Development and Differentiation.* p341-364. Edited by Ball, M. and Billett, F.S. Cambridge University Press, Cambridge, UK.

Hart, A.H., Corrick, C.M., Tymms, M.J. Hertzog, P.J. and Kola, I. (1995). Human ERG is a proto-oncogene with mitogenic and transforming activity. Oncogene 10, 1423-1430.

Heard, J.M., Roussel, M.F., Rettenmier, C.W. and Sherr, C.J. (1987). Multilineage hematopoietic disorders induced by transplantation of bone marrow cells expressing the v-fms oncogene. Cell 51, 663-673.

Hipskind, R.A., Rao, V.N., Mueller, C.G.F., Reddy, E.S.P. and Nordheim, A. (1991). Ets-related protein elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature 354, 531-534.

Hoatlin, M.E., Kozak, S.L., Lilly, F., Chakraborti, A., Kozak, C.A. and Kabat, D. (1990). Activation of erythropoietin receptors by Friend viral Gp55 and by erythrpoietin and down-modulation by the murine Fc-2^R resistance gene. Proc. Natl. Acad. Sci. USA 87, 9985-9989.

Hockenbery, D.M., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990). Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 348, 334-336.

Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M. and Korsmeyer, S.J. (1991). Bcl-2 protein is topographically restricted in tissues characterised by apoptotic death. Proc. Natl. Acad. Sci. USA 88, 6961-6965.

Hockenbery, D.M., Oltvai, Z.N. Yin, X.M., Milliman, C.L. and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75, 241-251.

Hockenbery, D.M. (1995). Bcl-2, a novel regulator of cell death. BioEssays 17, 631-638.

Hodge, D.R., Robinson, L., Watson, D., Lautenberger, J., Zhang, S.K., Venanzoni, M. and Seth, A. (1996). Interaction of ETS-1 and ERGB/FLI-1 proteins with DNA is modulated by spacing between multiple binding sites as well as phosphorylation. Oncogene 12, 11-18.

Hromas, R. and Klemsz, M (1994). The Ets oncogene family in development, proliferation and neoplasia. Int. J. Hematology 59, 257-265.

Hsu, H.L., Cheng, J.T., Chen, Q. and Baer, R. (1991). Enhancer-binding activity of the tal-1 oncoprotein in association with the E47/E12 helix-loop-helix proteins. Mol. Cell Biol. 11, 3037-3042

Hume, D.A., Yue, X., Ross, I.L., Favot, P., Lichanska, A. and Ostrowski, M.C. (1997). Regulation of CSF-1 receptor expression. Mol. Reprod. & Develop. 46, 46-54.

Humphries, R.K., Eaves, A.C. and Eaves, C.J. (1979). Characterisation of a primitive erythropoietic progenitor found in mouse marrow before and after several weeks in culture. Blood 53, 746-754.

Itoh, K., Ono, K., Sawada, H., Tezuka, H., Sakoda, H., Nakane, H., Uchiyama, T., Uchino, H. and Mori, K.J. (1988a). Establishment and characterisation of a transplantable erythrobalstic leukaemia in C3H mice. Leukaemia Res. 12, 471-478.

Itoh, K., Sasaki, R., Ono, K., Tezuka, H., Sakoda, H., Hitomi, K., Nakane, K., Uchitama, T., Uchino, H. and Mori, K.J. (1988b). Stromal cell dependent growth of the leukaemic cells from murine erythroblastic leukaemia. Jpn. J. Cancer Res. 9Gann) 79, 931-935.

Itoh, K., Tezuka, H., Sakoda, H., Konno, M., Nagata, K., Uchiyama, T., Uchino, H. and Mori, K.J. (1989). Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Exp. Hematol. 17, 145-153.

Janknecht, R., Monte, D., Baert, J.L. and Delaunoit, Y. (1996). The Ets-related transcription factor ERM is a nuclear target of singalling cascades involving MAPK and PKA. Oncogene 13, 1745-1754.

Janowski,M., and Boniver, J. (1986). Radiation-induced lymphomas and leukemias. Leukaemia Res. 10, 875-878.

Jen, Y., Weintraub, H. and Bevezra, R. (1992). Overexpression of Id protein inhibits the muscle differentiation program - in vivo association of Id with E2A proteins. Genes Dev. 6, 1466-1479.

Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Gralla, E.B., Valentine, J.S., Ord, T. and Bredesen, D.E. (1993). Bcl-2 inhibition of neural death, decreased generation of reactive oxygen species. Science 262, 1274-1277.

Kaya, M., Yoshida, K., Higashino, F., Mitaka, T., Ishii, S. and Fujinaga, K. (1996). A single ets-related transcription factor, E1AF, confers invasive phenotype on human cancer cells. Oncogene 12, 221-227.

Kinoshita, T., Yokota, T., Arai, K. and Miyajima, A. (1995). Regulation of Bcl-2 expression by oncogenic Ras protein in hematopoietic cells. Oncogene 10, 2207-2212.

Klemsz, M.J., McKercher, S.R., Celada, A., van Beveren, C. and Maki, R.A. (1990). The macrophage and B-cell specific transcription factor PU.1 is related to the ets oncogene. Cell 61, 113-124.

Klemsz, M.J., Maki, R.A., Papayannopoulou, T., Moore, J. and Hromas, R. (1993). Characterisation of the ets oncogene family member, fli-1. J. Biol. Chem. 268, 5769-5773.

Korsmeyer, S.J. (1995). Regulators of cell death. Trends in Genetics 11, 101-105.
Kozopas, K.M., Yang, T., Buchan, H.L., Zhou, P. and Craig, R.W. (1993). MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. Proc. Natl. Acad. Sci. USA 90, 3516-3520.

Kreider, B.L., Benezra, R., Rovera, G. and Kadesch, T. (1992). Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science 255, 1700-1702.

Kulessa, H., Frampton, J. and Graf, T. (1993). GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts and erythroblasts. Genes Dev. 9, 1250-1262.

Lacombe, C., Chretien, S., Lemarcharnel, V., Mayeux, P., Romeo, P.H., Gisselbrecht, S. and Cartron, J.P. (1991). Spleen focus-forming virus long terminal repeat insertional activation of the murine erythropoietin receptor gene in the T3CL-2 Friend-leukemia cell-line. J. Biol.Chem. 266, 6952-6956.

Lane, D. and Benchimol, S. (1990). P53 - oncogene or anti-oncogen? Genes Dev. 4, 1-8.

Lavigueur, A. and Bernstein, A. (1991). P53 transgenic mice - accelerated erythroleukemia induction by Friend-virus. Oncogene 6, 2197-2201.

Leroy-Viard, K., Vinit, M.-A., Lecointe, N., Jouault, H., Hibner, U., Romeo, P.-H. and Mathieu-Mahul, D. (1995). Loss of Tal-1 protein activity induces premature apoptosis of Jurkat leukemic T cells upon medium depletion. EMBO J. 14, 2341-2349.

Li, J.P., C'Andrea, A.D., Lodish, H.F., and Baltimore, D. (1990). Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature 343, 762-764. Green, A.R., et al. (1992). Oncogene 7, 653-660.

Lim, F. Kraut, N., Frampton, J. and Graf, T. (1992). DNA binding by c-Ets-1, but not v-Ets, is repressed by an intramolecular mechanism. EMBO J. 11, 642-652.

Lin, E.Y., Orlofsky, A., Berger, M.S. and Prystowsky, M.B. (1993). Characterisation of A1, a novel hemopoietic-specific early-response gene with sequence similarity to bcl-2. J. Immunol. 151, 1979-1988.

MacLeod, K., Leprince, D. and Stehelin, D. (1992). The ets gene family. Trends Biol. Sci. 17, 251-256.

Magnaghi-Jaulin, L., Masutani, H., Robin, P., Lipinski, M. and Harel-Bellan, A. (1996). SRE elements are binding sites for the fusion protein EWS-FLI-1. Nucleic Acid Res 24, 1052-1058.

Marais, R., Wynne, J., Treisman, R. (1993). The SRF accessory protein elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73, 381-393.

Marin, M.C., Fernandez, A., Bick R.J., Brisbay, S., Buja, L.M., Snuggs, M., McConkey, D.J., von Eschenbach, A.C., Keating, M.J. and McDonnell, T.J. (1996). Apoptosis suppression by bcl-2 is correlated with the regulation of nuclear and cytoxolic Ca2+. Oncogene 12, 2259-2266.

Marthinuss, J., Lawrence, L. and Seilberg, M. (1995). Apoptosis in Pam212, an epidermal keratinocyte cell line: a possible role for bcl-2 in epidermal differentiation. Cell Growth & Differentiation 6, 239-250.

Martin, D. and Orkin, S.H. (1990). Transcriptional activation and DNA-binding by the erythroid factor GF-1/NF-E1/Eryl. Genes Dev. 4, 1886-1898.

Martin, S.J. and Green, D.R. (1995). Protease activation during apoptosis: death by a thousand cuts? Cell 82, 349-352.

McDonald, J., Beru, N. and Goldwaser, E. (1987). Rearrangement and expression of erythropoietin genes in transformed mouse cells. Mol. Cell. Biol. 7, 365-370.

Meeker, T.C., Hardy, D., Willman, C., Hogan, T. and Abrams, J. (1990). Activation of the interleukin-3 gene by chromosome translocation in acute lymphocytic leukemia with eosinophilia. Blood 76, 285-289.

Merika, M. and Orkin, S.H. (1995). Functional synergy and physical interactions of the erythroid transcription factor GATA-1 with the Krüpple family proteins Sp1 and EKLF. Mol. Cell. Biol. 15, 2437, 2447.

Marvel, J., Perkins, G.R., Lopez Rivas, A. and Collin, M.K.L. (1994). Growth factor starvation of bcl-2 overexpressing murine bone marrow cells induced refractoriness to IL-3 stimulation of proliferation. Oncogene 9, 1117-1122.

McConkey, D. and Orrenius, S. (1996). Signal transduction pathways in apoptosis. Stem Cells 14, 619-631.

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H., Klemsz, M., Feeney, A.J., Wu, G.E., Paige, C.J. and Maki, R.A. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J. 15, 5647-5658.

Melet, F., Motro, B., Rossi, D.J., Zhang, L., Bernstein, A. (1996). Generation of a novel Fli-1 protein by gene targeting leads to a defect in thymus development and a delay in Friend virus-induced erythroleukaemia. Mol. Cell. Biol. 16, 2708-2718.

Miyashita, T., Harigai, M., Hanada, M. and Reed, J.C. (1994a). Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Res. 54, 3131-3135.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B. and Reed, J.C. (1994b). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene 9, 1799-1805.

Miyashita, T., Harigai, M., Hanada, M. and Reed, J.C. (1994c). Identification of a p53-dependent negative response element in the bcl-2 gene. Cancer Research 54, 3131-3135.

Miyazaki, T., Liu, Z.J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E.L., Perlmutter, R.M. and Taniguchi, T. (1995). Three distinct IL-2 signalling pathways mediated by bcl-2, c-myc and lck cooperate in hematopoietic cell proliferation. Cell 81, 223-231.

Mole, R.H. (1986). Radiation-induced acute myeloid leukemia in the mouse: experimental observations in vivo with implications for hypotheses about the basis of carcinogenesis. Leukemia Res. 10, 859-865.

Moreau-Gachelin, F., Tavitian, A., and Tambourin, P. (1988). Spi-1 is a putative oncogene in viraly induced murine erythroleukemias. Nature 331, 277-280.

Moreau-Gachelin, F., Ray, D., De Both, N.J., van der Feltz, M.J.M., Tambourin, P. and Tavitian, A. (1990). Spi-1 oncogene activation in Rauscher and Friend murine virus-induced acute erythroleukemias. Leukemia 4, 20-23.

Murakami, K., Mavrothalassitis, G., Bhat, N.K., Fisher, R.J. and Papas, T.S. (1993). Human ERG-2 protein is a phosphorylated DNA-binding protein - a distinct member of the ets family. Oncogene 8, 1559-1566.

Murrell, A.M., Bockamp, E.O., Gottgens, B., Chan, Y.S., Cross, M.A., Heyworth, C.M. and Green, A.R. (1995). Discordant regulation of SCL-Tal-1 mRNA and protein during erythroid differentiation. Oncogene 11, 131-139.

Muthusamy, N., Barton, K. and Leiden, J.M. (1995). Defective activation and survival of T cells lacking the Ets-1 transcription factor. Nature 377, 639-642.

Negrini, M., Silini, E., Kozak,C., Tsujimoto, Y. and Croce, C.M. (1987). Molecular analysis of mbcl-2: structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma. Cell 49, 455-463.

Nerlov, C., De Cesare, D., Pergola, F., Caracciolo, A., Blasi, F., Johnsen, M. and Verde, P. (1992). A regulatory element that mediates cooperation between a PEA3-AP-1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. EMBO J. 11, 4573-4582.

Nibbs, R.J.B., Itoh, K., Ostertag, W. and Harrison, P.R. (1993). Differemtiation arrest and stromal cell-independent growth of murine erythroleukaemia cells are associated with elevated expression of ets-related genes but not with mutation of p53. Mol. Cell. Biol. 13, 5582-5592.

Nunez, G. and Clarke, M.F. (1994). The Bcl-2 family of proteins: regulators of cell death and survival. Trends Cell Biol. 4, 399-403.

Nunn, M.F., Seeberg, P.H., Moscovici, C. and Duesberg, P.H. (1983). Tripartite structure of the avian erythroblastosis virus-E26 transforming gene. Nature 306, 391-395.

Ohagan, R.C., Tozer, R.G., Symons, M., McCormick, F. and Hassell, J.A. (1996). The activity of the Ets transcription factor PEA3 is regulated by two distinct MAPK cascades. Oncogene 13, 1323-1333.

Ohneda, O., Yania, N. and Obinata, M. (1990). Microenvironment created by stromal cells is essential for a rapid expansion of erythroid cells in mouse fetal liver. Development 110, 379-384.

Oltvai, Z., Milliman, C. and Korsmeyer, S.J. (1993). Bcl-2 heterodimerises in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74, 609-619.

Ono, K., Barre-Sinoussi, F., Rey, F., Jasmin, C. and Chermann, J.C. (1984). Organ distribution of reverse transcriptase and cellular DNA polymerases in Friend virus-infecred mice: analysis by phosphocellulose column chromatography. Cell Molec. Biol. 30, 303-309.

O'Prey, J., Ramsay, S., Chambers, I. and Harrison, P.R. (1993). Transcriptional up-regulation of the mouse cytosolic glutathione peroxidase gene in erythroid cells is due to a tissue-specific 3' enhancer containing functionally important CACC/GT motifs and binding sites for GATA and Ets transcription factors. Mol. Cell. Biol. 13, 6290-6302.

Orkin, S.H. (1995). Hematopoiesis: how does it happen? Curr. Opion. Cell. Biol. 7, 870-877.

Otani, H., Erdos, M. and Leonard, W.J. (1993). Tyrosine kinase(s) regulate apoptosis and bcl-2 expression in a growth factor-dependent cell line. J. Biol. Chem. 268, 22733-22736.

Papadopoulos, P., Ridge, S.A., Boucher, C.A., Stocking, C. and Wiedemann, L.M. (1995). The novel activation of ABL by fusion to an ets-related gene, TEL. Cancer Res. 55, 34-78.

Papavassiliou, A.G. (1994). 1,10-Phenanthroline-copper ion nuclease footprinting of DNA-protein complexes in situ following mobility-shift electrophoresis assays. Kneale, G.G. (ed.), Methods in Molecular Biology, 30. Human Press Inc., Totowa, N.J., USA

Pevny, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.F., D'Agati, V., Orkin, S.H. and Costantini, F. (1991). Erythroid differentiation in chimeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 349, 257-260.

Pevny, L., Lin, C.S., D'Agati, V., Simon, M.C., Orkin, S.H. and Costantini, F. (1995). Development of hemotopoietic cells lacking transcription factor GATA-1. Development 121, 163-172.

Plumb, M.A. and Goodwin, G.H. (1988). Detection of sequence-specific protein-DNA interactions by the DNA footprinting technique. J.M. Walker (ed.), Methods in Molecular Biology, 4. Human Press Inc., Clfton, N.J., USA

Plumb, M.A., Frampton, J., Wainwright, H., Walker, M., MacLeod, K., Goodwin, G. and Harrison, P.R. (1989). GATAAG: a cis-control region binding an erythroid-specific nuclear factor with a role in globin and non-globin gene expression. Nucleic Acids Res. 17, 73-92.

Pongubala, J.M.R., Beveren, C.V., Nagulapalli, S., Klemsz, M.J., McKercher, S.R., Maki, R.A. and Atchison, M.L. (1993). Effect of PU.1 phorphorylation on interaction with NF-EM5 and transcriptional activation. Science 259, 1622-1625.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F.W. and Orkin, S.H. (1996). The T cell leukemia oncoprotein SCL-tal-1 is essential for development of all hematopoietic lineages. Cell 86, 47-57.

Prasad, D.D.K., Rao, V.N., Lee, L., and Reddy, E.S.P. (1994). Differentially spliced erg-3 product funstions as transcriptional activators. Oncogene 9, 669-673.

Prasad, K.S.S., Jordan, J.E., Koury, M.J., Bondurant, M.C. and Brandt, S.J. (1995). Erythropoietin stimulates transcription of the TAL-1/SCL gene and phorphorylation of its protein products. J. Biol. Chem. 270, 11603-11611.

Quesenberry, P.J. (1992). Stroma-dependent hematolymphopoietic stem cells. In *Hematopoietic stem cells: animal models and human transplantation.* Muller-Sieburg, C., Torok-Storb, B. Visser, J. and Storb, R. (eds). Spring-Verlag, Berlin. p151-166.

Rabault, B., Roussel, M.F., Quang, C.T. and Ghysdael, J. (1996). Phosporylation of Ets-1 regulates the complementation of a CSF-1 receptor impaired in mitogenesis. Oncogene 13, 877-881.

Rao, V.N., Papas, T.S. and Reddy, E.S.P. (1987). Erg, a human ets-related gene on chromosome 21: alternative splicing, polyadenylation and translation. Science 237, 635-639.

Rao, V.N., Ohno, T., Prasad, D.D.K., Bhattacharya, G. and Reddy, E.S. (1993a). Analysis of the DNA-binding and transcriptional activation functions of human Fli-1 protein. Oncogene 8, 2167-2173.

Rao, V.N. and Reddy, E.S.P. (1993b). Elk-1 proteins are phosphoproteins and activators of mitogen-activated protein kinase. Cancer Res. 53, 3449-3454.

Rao, V.N. and Reddy, E.S.P. (1994). Elk-1 proteins interact with MAP kinase. Oncogene 9, 1855-1860.

Reddy, E.S.P., V.N. Rao and Papas, T.S. (1987). The erg gene: a human gene related to the ets oncogene. Proc. Natl. Acad. Sci. USA 84, 6131-6135.

Reddy, E.S.P. and Rao, V.N. (1991). Erg, an ets-related gene, codes for sequence-specific transcriptional activators. Oncogene 6, 2285-2289.

Reed, J.C., Haldar, S., Croce, C.M. and Cuddy, M.P. (1990). Complementation by Bcl-2 and c-Ha-Ras oncogenes in malignant transformation fo rat embryo fibroblasts. Mol. Cell. Biol. 10, 4370-4374.

Reed, J.C. (1994). Bcl-2 and the regulation of programmed cell death. J. Cell Biol. 124, 1-6.

Ridge, S.A., Worwood, M., Oscier, D., Jacob, A. and Padua, R.A. (1990). Fms mutations in myelodysplastic, leukemic and normal subjects. Proc. Natl. Acad. Sci. USA 87, 1377-1380.

Rinaudo, M.S., Su, K., Falk, L.A., Haldar, S. and Mufson, R.A. (1995). Human Interleukin-3 receptor modulates bcl-2 mRNA and protein levels through protein kinase C in TF-1 cells. Blood 86, 80-88.

Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R. P., Metcalf, D. and Begley, C.G. (1995a). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Proc. Natl. Acad. Sci. USA 92, 7075-7079.

Robb, L., Rasko, J.E.J., Bath, M.L., Strasser, A. and Begley, C.G. (1995b). Scl, a gene frequently activated in human T cell leukaemia, does not induce lymphomas in transgenic mice. Oncogene 10, 205-209.

Robb, L., Elwood, N.J., Elefanty, A.G., Kontgen, F., Li, R., Barnett, L.D. and Begley, C.G. (1996). The scl gene product is required for the generation of all hematopoietic lineages in the adult mouse. EMBO J. 15, 4123-4129.

Roussel, M.F., Davis, J.N., Cleveland, J.L., Ghysdael, J. and Hiebert, S.W. (1994). Dual control of myc expression through a single DNA binding site targeted by ets family proteins and E2F-1. Oncogene 9, 405-415.

Rowan, S. and Fisher, D.E. (1997). Mechanisms of apoptotic cell death. Leukemia 11, 457-465.

Ruscetti, S.K., Janesch, N.K., Chakroborti, A., Sawyer, S.T. and Hankins, W.D. (1990). Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. J. Virol. 63, 1057-1062.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Mannual.* 2nd edition, Cold Spring Harbor laboratory Press, Cold Spring Harbor, USA.

Savill, J., Fadok, V. and Henson, P. (1993). Phagocytic recognition of cells undergoing apoptosis. Immunol. Today 14, 131-136.

Sawada, H., Tezuka, H., Kirikae, T., Yoshida, Y., Uchino, H. and Mori, K.J. (1986). Physiopathological studies on granulocyte-macrophage colony stimulating factor and multi colony stimulating factor producing leukemia, L8313, induced by irradiation of C3H mice. Leukaemia Res. 10, 1461-1468.

Sawada, K., Krantz, S.B., Dessypris, E.N., Koury, S.T. and Sawyer, S.T. (1989). Human colony forming units-erythroid do not require accessory cells, but do require direct interaction with insulin-like growth factor I and/or insulin for erythroid development. J. Clin. Invest. 83, 1701-7109.

Sawada, K., Krantz, S.B., Dai, C.H., Sata, N., Ieko, M., Sakurama, S., Yasukouchi, T. and Nakagawa, S. (1991). Transitional change of colony stimulating factor requirements for erythroid progenitors. J. Cell Physiol. 149, 1-8.

Sawyer, C.L., Denny, C.T. and Witte, O.N. (1991). Leukaemia and the desruption of normal hematopoiesis. Cell 64, 337-350.

Seth, A., Robinson, L., Thompson, D.M., Watson, D.K. and Papas, T.S. (1993). Transactivation of GATA-1 promoter with ETS1 and ETS2 and ERG/Hu-FLI-1 proteins: stabilisation of the ETS1 protein binding on GATA-1 promoter sequences by monoclonal antibody. Oncogene 8, 1783-1790.

Seto, M., Jaeger, U., Hockett, R.D., Graninger, W., Bennett, S., Goldman, P. and Korsmeyer, S.J. (1988). Alternative promoters and exons, somatic mutation and deregulation of the bcl-2-Ig fusion gene in lymphoma. EMBO J. 7, 123-131.

Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994). Science 265, 1573-1577.
Seth, A., Ascione, R., Fisher, R.J., Mavrothalassitis, F.J., Bhat, S.K. and Papas, T.S. (1992). The ets gene family. Cell Growth & Differentiation ?, 327-334.

Sgouras, D.N., Athanasiou, M.A., Beal, G.J., Fisher, R.J., Blair, D.G. and Mavrothalassitis, G.J. (1995). ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress ets-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. EMBO J. 14, 4781-4793.

Shapiro, L.H. (1995). Myb and Ets proteins cooperate to transactivate an early myeloid gene. J. Biol. Chem. 270, 8763-8771.

Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T. and Stanley, E.R. (1985). The c-fms oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41, 665-676.

Shimizu, K., Ichikawa, H., Tojo, A., Kaneka, Y., Maseki, N., Hayashi, Y., Ohira, M., Asano, S. and Ohki, M. (1993). An ets-related gene, ERG, is rearranged in human myeloid leukemia with t(16;21) chromosomal translocation. Proc. Natl. Acad. Sci. USA 90, 10280-10284.

Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H. and Tsujimoto, Y. (1996). Bcl-2 expression prevents activation of the ICE protease cascade. Oncogene 12, 2251-2257.

Shivdasani, R.A., Mayer, E.L., and Orkin, S.H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 373, 432-434.

Shivdasani, R.A. and Orkin S.H. (1996). The transcriptional control of hematopoiesis. Blood 87, 4025-4039.

Shoji, W., Yamamoto, T. and Obinata, M. (1994). The helix-loop-helix protein Id inhibits differentiation of murine erythroleukemia cells. J. Biol. Chem. 269, 5078-5084.

Shurtleff, S.A., Buijs, A., Behm, F.G., Rubnitz, J.E., Raimondi, S.C., Hancock, M.L., Chan, G.C.F., Pui, C.H., Grosveld, G. and Downing, J.R. (1995). TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis.

Silver, J. and Teich, N. (1981). Expression of resistance to Friend virus-stimulated erythropoiesis in bone-marrow chimeras containing FV-2^{RR} and FV-2^{SS} bone-marrow. J. Exp. Med. 154, 126-137.

Siddique, H.R., Rao, V.N., Lee, L. and Reddy, E.S.P. (1993). Characterisation of the DNA-binding and transcriptional activation domains of the erg protein. Oncogene 8, 1751-1755.

Sieweke, M.H., Tekotte, H., Frampton, J. and Graf, T. (1996). MafB is an interaction partner and repressor of Ets-1 that inhibits erythroid differentiation. Cell 85, 49-60.

Simon, M.C., Pevny, L., Wiles, M.V., Keller, G., Costantini, F. and Orkin, S.H. (1992). Rescue of erythroid development in gene targeted GATA-1-mouse embryonic stem cells. Nat. Genet. 1, 92-98.

Sorensen, P., Lessnick, S.L., Lopez-Terrada, D., Liu, X.F., Triche, T.J. and Denny, C.T. (1994). A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat. Genet. 6, 146-151.

Strasser, A., Harris, A.W., Bath, M.L. and Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348, 331-333.

Strasser, A., Whittingham, S., Vaux, D.L., Bath, M.L., Adams, J.M., Cory, S., and Harris, A.W. (1991). Enforced BCL2 expression of B-lympoid cells prolons antibody responses and elicits autoimmune disease, Proc. Natl. Acad. Sci. USA 88, 8661-8661.

Suzuki, J., Fujita, J., Taniguchi, S., Suginoto, K. and Mori, K.J. (1992). Characterisation of murine hemopoietic supportive (MS-1 and MS-5) and non-supportive (MS-K) cell lines. Leukemia 6, 452-458.

Tanigawa, T., Elwood, N., Metcalf, D., Cary, D., Deluca, E., Nicola, N.A. and Begley, C.G. (1993). The scl gene-product is regulated by and differentially regulates cytokine responses during myeloid leukemic-cell differentiation. Proc. Natl. Acad. Aci. USA 90, 7864-7868.

Tsai, S.F., Martin, D.I.K., Zon, L.I., D'Andrea, A.D., Wong, G.G. and Orkin, S.H. (1989). Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature 339, 446-451.

Tsujimoto, Y., Cossman, E., Iaffe, E. and Croce, C.M. (1985). Involvement of the bc-2 gene in human follicular lymphoma. Science 228, 1440-1443.

Urness, L.D. and Thummel, C.S. (1990). Molecular interactions within the ecdysone regulatory hierarchy: DNA binding porperties of the *Drosophila* ecdysone-inducible E74A proteins. Cell 63, 47-61.

Valge-Archer, V.E., Osada, H., Warren, A.J., Forster, A., Li, J., Baer, R. and Rabbits, T.H. (1994). The LIM protein RBTN2 and the basic helix-loop-helix protein TAL1 are present in a complex in erythroid cells. Proc. Natl. Acad. Sci. USA 91, 8617-8621.

Vaux, D.L., Cory, S. and Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperation with c-myc to immortalise pre-B cells. Nature 335, 440-442.

Vaux, D.L., Weissman, I.L. and Kim, S.K. (1992). Prevention of programmed cell death in Caenorhabditis elegans by human bcl-2. Science 258, 1955-1957.

Veis-Novack, D.J., Sorenson, C.M., Shutter, J.R. and Korsmeyer, S.J. (1993). The Bcl-2 deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75, 229-240.

Veis-Novack, D.J. and Korsmeyer, S.J. (1994). Bcl-2 protein expression during murine development. Am. J. Path. 145, 61-73.

Voronova, A.F. and Lee, F. (1994). The E2A and tal-1 helix-loop-helix proteins associate in vivo and are modulated by Id proteins during interleukin 6-induced myeloid differentiation. Proc. Natl. Acad. Sci. USA 91, 5952-5956.

Wadman, I., Li, J., Bash, R.O., Forster, A., Osada, H., Rabbits, T.H. and Baer, R. (1994). Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. EMBO J. 13, 4831-4839.

Wang, C. Petryniak, B., Thompson, C., Kaelin, W. and Leiden, J.M. (1993). Regulation of the ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science 260, 1330-1335.

Wang, C.Y., Bassuk, A.G., Boise, L.H., Thompson, C.B., Bravo, R. and Leiden, J.M. (1994). Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperatinve binding of Elf-1 and AP-1 transcription factors. Mol. Cell. Biol. 14, 1153-1159.

Warren, A.J., Colledge, W.H., Carlton, M.B.L., Evans, M.J., Smith, A.J.H. and Rabbits, T. H. (1994). The oncogenic cysteine-rich LIM domain protein Rbtn2 is essential for erythroid development. Cell 78, 45-57.

Watson, D.K., Smyth, F.E., Thompson, E.M., Cheng, J.Q., Testa, J.R., Papas, T.S. and Seth, A. (1992). The ERGE/Fli-1 gene: isolation and characterisation of a new member of the family of human ETS transcription factors. Cell Growth & Differentiation 3, 705-713.

Watson, D.K., Robinson, L., Hodge, D.R., Kola, I., Papas, T.S. and Seth, A. (1997). TLI1 and EWS-FLI-1 function as ternary complex factors and ELK1 and SAP1α function as ternary and quaternary complex factors on the Egr1 promoter serum response elements. Oncogene 14, 213-221.

Wasylyk, B., Wasylyk, C., Flores, P., Begue, A., Lerprince, D. and Stehelin, D. (1990). The c-ets protooncogens encode transcription factors that cooperate with c-fos and c-jun for transcription activation. Nature 346, 191-193.

Wasylyk, C., Gutman, A., Nicholson, R. and Wasylyk, B. (1991). Eth c-ets oncoprotein activates and stromelysin promoter through the same elements as several non-nuclear oncoproteins. EMBO J. 10, 1127-1134.

Wasylyk, B., Hahn, S.L. and Giovane, A. (1993). The Ets family of transcription factors. Eur. J. Biochem. 211, 7-18.

Wasylyk, C., Maria, S.-M., Sobieszczuk, P. and Wasylyk, B. (1994). Reversion of Ras transformed cells by Ets transdominant mutants. Oncogene 9, 3665-3673.

Wasylyk, C., Bradford, A.P., GutierrezHartmann, A. and Wasylyk, B. (1997). Conserved mechanisms fo Ras regulation of evolutionary related transcription factor, Ets1 and pointed P2. Oncogene 14, 899-913.

Weber, M.C. and Tykocinski, M.L. (1994). Bone marrow stromal cell blockade of human leukemic cell differentiation. Blood 83, 2221-2229.

Weiss, M.J., Keller, G. and Orkin, S.H. (1994). Novel insights into erythroid development revealed through in vitro differentiation of GATA-1-embryonic stem cells. Genes Dev. 8, 1184-1197.

Weiss, M. and Orkin, S.H. (1995). Transcription factor GATA-1 permits survival and maturation of erythroid precursors by preventing apoptosis. Proc. Natl. Acad. Aci. USA 92, 9623-9627.

Whetton, A.D. and Dexter, T.M. (1993). Influence of growth factors and substrates on differentiation of haemopoietic stem cells. Curr. Opion. Cell Biol. 5, 1044-1049.

Wilkinson, D.A., Neale, G.A.M., Mao, S., Naeve, C.W. and Goorha, R.M. (1997). Elf-2, a rhombotin-2 binding ets transcription factor: discovery and potential role in T cell leukaemia. Leukemia 11, 86-96.

Wu, H., Moulton, K., Horval, A., Parik, S. and Glass, C.K. (1994). Combinatorial interactions between AP-1 and ets domain proteins contribute to the developmental regulation of the macrophage scavenger receptor gene. Mol. Cell. Biol. 14, 2129-2139.

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1980), Cell death: the significance of apoptosis. Int. Rev. Cytol. 68: 251-305.

Yamada, T., Kondoh, N., Matsumoto, M., Yoshida, M., Maekawa, A. and Oikawa, T. (1997). Overexpression of PU.1 induces growth and differentiation inhibition and apoptotic cell death in murine erythroleukaemia cells. Blood 89, 1383-1393.

Yanai, N., Matsuya, Y. and Obinata, M. (1989). Spleen stromal cell lines selectively support erythroid colony formation. Blood 74, 2391-2397.

Yanai, N., Satoh, T. and Obinata, M. (1991). Endothelial cells create a hematopoietic inductive microenvironment preferential to erythropoiesis in the mouse spleen. Cell Structure and Function 16, 87-93.

Yanai, N., Sekine, C., Yagita, H. and Obinata, M. (1994). Roles for integrin very late activation antigen-4 in stroma-dependent erythropoiesis. Blood 83, 2844-2850.

Yang, B.S., Hauser, C.A., Henkel, G., Colman, M.S., Vanbeveren, C., Stacey, K.J., Hume, D.A., Maki, R.A. and Ostrowski, M.C. (1996). Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets-1 and c-Ets-2. Mol. Cell. Biol. 16, 538-547.

Yin, X.M., Oltvai, Z.N. and Korsmeyer, S.J. (1994). BH1 and BH2 domains of bcl-2 are required for inhibition of apoptosis and heterodimerisation with Bax. Nature 369, 321-323.

Young, R.L. and Korsmeyer, S.J. (1993). A negative regulatory element in the bcl-2 5'-untranslated region inhibits expression from an upstream promoter. Mol. Cell. Biol. 13, 3686-3697.

Zhang, L., Lemarchandel, V., Romeo, P.-H., Ben-David, Y., Greer, P. and Bernstein, A. (1993). The Fli-1 proto-oncogene, involved in erythroleukemia and Ewing's sarcoma, encodes a transcriptional activator with DNA-binding specificities distinct from other Ets family members. Oncogene 8, 1621-1630.

Zhang, L., Eddy, A., Teng, Y.-T., Fritzler, M., Kluppel, M., Melet, F. and Bernstein, A. (1995). An immunological renal disease in transgenic mice that overexpress Fli-1, a member of the ets family of transcription factor genes. Mol. Cell. Biol. 15, 6961-6070.

Zhang, K.Z., Westberg, J.A., Holtta, E. and Andersson, L.C. (1996). BCL-2 regulates neural differentiation. Proc. Natl. Acad. Sci. USA 93, 4504-4508.

Zipori, D. (1992). The renewal and differentiation of hemapoietic stem cells. FESEB J. 6, 2691-2697.

Zipori, D. and Tamir, M. (1989). Stromal cells of hemopoietic origin. Int. J. Cell Cloning 7, 281-291.